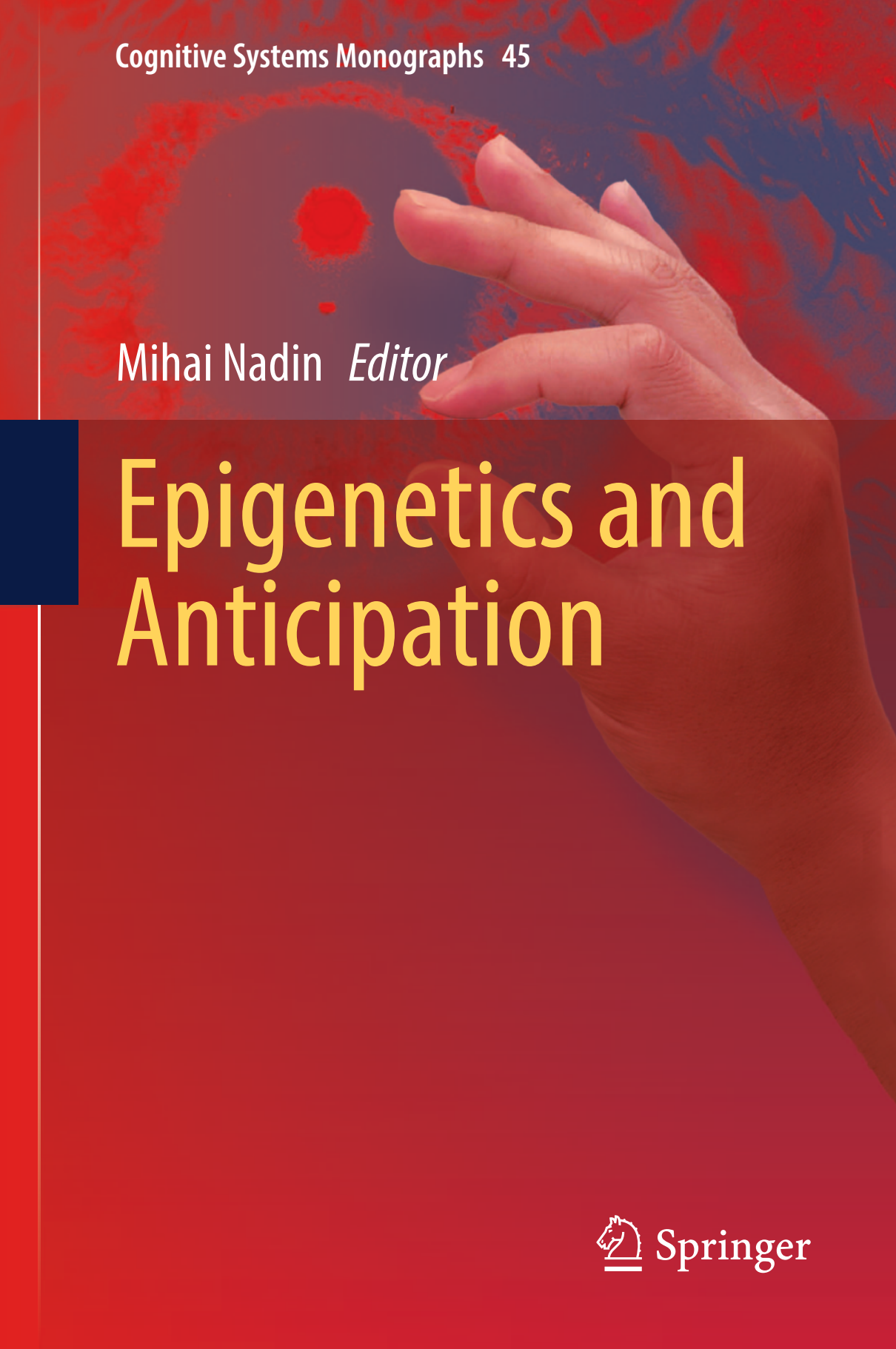
Cognitive Systems Monographs 45



Mihai Nadin *Editor*

Epigenetics and Anticipation

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Volume 45

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Epigenetics and Anticipation

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Contents

Context—A Subject More Important Than Ever

Mihai Nadin

It was supposed to take place in Delmenhorst (Germany) at the Hanse Wissenschaftskolleg (Institute for Advanced Study) in May (10–12) of 2021. Almost 40 scientists from around the world submitted proposals. Springer Publishers committed to a book supposed to be printed at the end of 2021 or spring of 2022. And then came Covid-19. The World Health Organization reported (in May of 2021) 5.5 million cases; almost 100,000 died as that time. It was only the beginning. As undesired as this pandemic was (and still is), it became a test of a number of various assumptions—including those related to in-person conferences. Leaving aside economic, social, political, racial, ethical, and other considerations—all of extreme significance—it is clear that science itself underwent a major experiment. Genetics played a spectacular role: millions of all kinds of sequencing operations were carried out. Epidemiology scored also high. Despite spectacular technical performance, and despite the heroic efforts of many practicing physicians, it is quite evident that science failed to prevent the disaster, not to say to properly address it. Of course, substantiating a value judgment as radical as the one I just articulated would take more than some introductory lines to this volume. (Actually, I dedicated a whole book to the subject). The pandemic became *volens-nolens* the opportunity to frame the subject of this publication: Anticipation and Epigenetics.

The crisis triggered by the SARS-CoV-2 virus evinced the significance of epigenetic inheritance. Covid-19 is the outcome of the processes through which epigenetic inheritance takes place. It is also the direct result of lack of prevention: that is, anticipatory actions that could have spared humankind the terrible consequences of the pandemic (by now the number of those who lost their life is close to 12 million). In other words, what had been the subject that the planned conference would have

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discussed in the comfort of academic debate became a reality. Moreover, given everything that the scientific community contributed in confronting the crisis, there is an urgency in providing not so much an analysis of the not yet settled past, but a perspective: What could the epigenetic consequences of the mRNA-based vaccines be? What are the implications of post-antiviral medication induced infections (i.e., treatments that in some cases lead to relapse)? More important, what is the connection between anticipatory actions expressed in the current research for vaccines and new therapies, and the long-term consequences of such actions?

Even the examination of this broad perspective does not yet define the context. Solid research, dating back to the end of the 1990s (cf. Petronis et al. 1997) focused on a specific condition—in which symptoms tend to become more severe, and appear at an earlier age. The question of whether this particular condition defined as anticipation is the consequence of genetic or epigenetic processes remained open. In some ways, a short communication such as the one mentioned, becomes part of what the conference, that could not take place as in-person event, would have been. Therefore, the text and the short position statement of 2 of the authors became part of this volume.

It is worth mentioning that the subject matter of epigenetics and of anticipation share in the rather difficult acceptance process through which the scientific community validates a new perspective. However, epigenetics fared so far better than anticipation, mostly because genetics—to which it was initially reduced—proved such a spectacular knowledge domain. This was not the case with anticipation—its history is less well defined, and its role in science remains a subject of debate. Therefore, the Study Group Anticipation Across Disciplines hosted by the Institute for Advances Studies, proved to be from its start of extreme significance to those trying to articulate a coherent perspective of the role of anticipatory processes. Indeed, with anticipation as a subject, several conferences took place, to which previous volumes were dedicated (*Anticipation and Medicine,* 2016; *Anticipation Across Disciplines*, 2015; *Learning from the Past. Early Soviet/Russian contributions to a science of anticipation*). It is in this concrete manner that a new horizon was defined and a foundation for scientific debate was made available. Let me quote from the application for funding submitted to the German Science Foundation:

> The scientific objective of the workshop "Epigenetic and Anticipation" is to transform the awareness of the anticipatory perspective into actionable methods (for practitioners of medicine, but also for those who design new technologies, or who advocate sustainable alternatives). Solutions that are reactive in nature will not do. Anticipatory awareness means the realization that reductionism—focus on one aspect to the detriment of the larger understanding of reality—undermines our effort in addressing issues of ecology, health, scientific and technological progress. For example, a pharmaceutical company will try to develop a drug that will reverse an epigenetically expressed condition, such as cancers. The anticipatory approach will translate into being aware of factors that could have harmful epigenetic effects, and help avoiding them.

The proposal did not predict the pandemic, although it expressed ideas that became critical during the pandemic. It reiterated the fact that during its still formative

years, anticipation ascertained a perspective complementary to that of reductionist-determinism. It also took note of the fact that genetics is grounded in reductionist-determinism, while epigenetics suggests an alternative view, while still seeking the certitude of the experimental method. I was honored to be joined by Prof. Dr. Kerstin Schill, Rector of the Hanse Institute for Advanced Study and by Dr. Dorothe Poggel, in charge of the Brain-Mind program as co-PI's for the proposal.

What in the final analysis justifies the investment (in time, dedication, research effort, funding) in producing the repository of a conference that reality rendered impossible is the trust and dedication of everyone who remained on board. First and foremost, the Hanse Institute for Advanced Study (Hanse Wissenschaft-skolleg/HWK). In particular, Dr. Reto Weiler and Dr. Dorothe Poggel never ceased to support the International Study Group in Anticipation Across Disciplines. The University of Texas at Dallas, by now a tier-1 institution, continued its support of the antÉ—Institute for Research in Anticipatory Systems for almost 18 years. This, as a virtual community of researchers, was able to address the foundation of a science of anticipation as well as applications ranging from motoric aspects of aging, brain plasticity, performance in critical contexts, creativity, etc. The Institute dedicated means to preparing this publication. When everyone was struck by the fears of the day—not to say directly affected by the pandemic—Springer Publishers, in particular Dr. Thomas Ditzinger, Editorial Director (Interdisciplinary Applied Sciences), understood that it takes longer to assemble a finalized set of papers during a pandemic than after a conference held under normal conditions. Without the support of everyone involved, this project would not have been brought to fruition. As Editor of this volume, I would like to express my gratitude to all the authors. The International Agency for Research on Cancer (IARC) of the World Health Organization announced, as we worked on this volume, that the inaugural recipient of the IARC Award for Women in Cancer Research is Dr. Cristina Stefan, Director of the Institute of Global Health Equity Research in Kigali, Rwanda. She is joined by a group of researchers from the University of Medicine in Cluj-Napoca in sharing insight into the relevance of epigenetics and anticipation in the treatment of cancer. Horst Horsthemke, active in the epigenetics community for a long time was exemplary in supporting the making of this book. Moshe Szyf, who, together with Michael Meaney is credited for having established the field of behavioral epigenetics, made an impressive effort to live up to a commitment made before the pandemic changed out lives. Actually, when the going gets tough—and tough it was—everyone who remains committed and never compromise integrity deserve recognition. Therefore, Dr. Asma Naz who gave the volume more than a formatting and Maryam Ashkaboosi, for many images, deserve no less than the scientists mentioned to be named for their contribution. May this volume lead to many discussions and follow-up research.

Environment and Genetic Processes

DNA Methylation as an Epigenetic Mechanism of Anticipation

Moshe Szyf

Abstract The genomes of species across the evolutionary landscape contain in addition to the genetic information encoded in the 4 letters of the DNA minor bases, methylated adenines and cytosines. In contrast to the genetic sequence which is copied from a template according to Watson and Crick rules with high fidelity base modification is catalyzed by an independent DNA methylation machinery by DNA methyltransferases that catalyze the transfer of methyl groups from the methyl donor S-adensoyl methionine to cytosine or adenine bases in DNA at specific sequence contexts. DNA methylation is the most proximal epigenetic mechanism to DNA and provides identical genomes with differential identities. This mechanism is used during development for providing cellular and tissue specific identity. DNA methylation exponentially expands the information content of DNA in space, time and experiential and environmental context. DNA methylation positions genes to be reactive to future endogenous and exogenous conditions or triggers and is thus an anticipatory mechanism encoded in our DNA. This review will discuss the basic mechanisms of DNA methylation and gene expression and how they anticipate downstream developmental ad environmental trajectories.

Keywords DNA methylation · Epigenetics · Early life stress · Anticipation · Chromatin · Trauma · DOHAD · Development

1 Introduction

The semiconservative replication of DNA across generations follows strict deterministic rules which allow the propagation and conservation of the integrity of genetic information across generations, within organisms and across the lifespan [1, 2]. The base composition of the template defines the sequence of the daughter strand; a thymidine is added across an adenine while a guanidine is added across a cytidine [2]. The same rules apply to transcription of messenger RNA from the gene which

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is eventually translated to proteins following the rules of the genetic code and thus defining the phenotype. This has led to the principal dogma of modern biology equating genotype with phenotype [3]. Since alterations in the genotype could lead to changes in the phenotype, several mechanisms of repair during and after replication have evolved to safeguard the integrity of the genome [4]. When small errors in this strict process are introduced in the germline either through small imperfections in the DNA replication machinery or through exposure to physical and chemical genotoxic agents, they might eventually lead to phenotypic changes that could be stabilized by natural selection driving intraspecies evolution as well as emergence of new species [5]. Adverse genetic changes lead to genetic disease and create interindividual phenotypic variation within the species that explains a significant part of the interindividual phenotypic variation [6]. Somatic errors in DNA replication can lead to cancer while programmed editing of DNA sequence is involved in generating diversity in the immune system antibody repertoire [7].

The strict inheritance of genetic information according to Watson and Crick rules within an organism results in a multicellular organism that contain billions of cells with identical genetic information [8]. Since in complex multicellular organisms the process of development involves differentiation of cells and their assembly into tissues and organs with numerous different phenotypes, other mechanisms must exist to confer differentiated identities to identical genetic sequences. This process has been defined by Waddington in the middle of the previous century as "epigenetics" [9]. A large body of biochemical mechanisms have been elucidated since then to be involved in "epigenetic programming" and they include chemical covalent modifications of the histone proteins which are the scaffolds of chromatin the form of compaction of DNA in the nucleus [10–13] as well as covalent chemical modification of the DNA molecule itself by enzymatic catalysis of transfer of methyl moieties from the methyl donor S-adenosylmethionine to the 5′ position on cytosines or 6′ positions on adenines [14–16]. Small and large noncoding RNAs [17–19] as well as chemical modification by methylation of mRNA and noncoding RNA [20] are also involved in cell type specific epigenetic programming of gene transcription and translation during cellular differentiation in development. The combinations of these mechanisms provide an enormous combinatorial repertoire of complexity that could explain the enormous heterogeneity of genomic functions across different organs, tissues and cells of an individual multicellular organism like ourselves [21–23]. Numerous studies have shown that genes acquire different epigenetic programs during development and that either genetic disruption of the genes encoding enzymes catalyzing epigenetic programming or pharmacological inhibition of these enzymes could lead to changes in genomic function, disrupt development and alter the phenotype [24–26].

This review will focus on DNA methylation, the most proximal epigenetic mechanism to DNA [27]. The DNA molecule itself contains both genetic information that is interpreted by the genetic code and epigenetic information in the form of methyl moieties covalently bound to DNA bases that shape the cellular identity. DNA methylation is the only epigenetic modification that could be studied in ancient organisms [28, 29] since it is part of the DNA chemical structure. Epigenetic modifications are highly corelated through biochemical linking mechanisms [30] such as

the targeting of DNMT3B to histone modifications at H3K9 [31] and DNMT3A to H3K36me2 [32] and recruitment of histone deacetylases complexes to methylated DNA through methylated DNA binding domain proteins (MBDs) [33], as well as recruitment of DNMTs by the histone methyltransferase EZH2 and the PRC complex [34]. Although the correlation of the different epigenetic mechanisms is imperfect and hence the combinatorial value of multiple epigenetic modifications, nevertheless recent approaches suggest that it is possible to impute DNA methylation profiles from other chromatin modifications and these methods are now used to impute missing DNA methylation data in genome wide sequencing and predicting enhancers and transcription factor binding using DNA methylation data [35].

2 DNA Methylation is a Covalent Epigenetic Modification of DNA

DNA methylation in vertebrates is catalyzed by DNA methyltransferases (DNMT) which transfer a methyl moiety from the methyl donor S-adenosylmethionine to the 5′ position on the cytosine ring in DNA [14, 16]. New DNA methylation events are catalyzed by de novo methyltransferases [36, 37] and the pattern of methylation that is formed is then replicated by a maintenance DNA methyltransferase 1 (DNMT1) [38]. This is possible since the sequence that is methylated by DNMT1 is CG which is a palindrome and the common methylated dinucleotide in vertebrate DNA [39], that is across a methylated CG dinucleotide on the parental strand there is a CG on the daughter strand. This enzyme could therefore faithfully copy methylation patterns because it has a strong preference to hemi-methylated CG substrate, which is generated when a methylated CG is replicated by the DNA polymerase machinery just before the nascent CG is methylated (see Fig. 1).

DNMT1 has very weak activity on a pair of CG dinucleotides that are unmethylated on both the parental and nascent strands [38], therefore it would not introduce new methylated positions (Fig. 1). DNMT1 is also guided to the replication fork by another protein ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) [40]. DNMT1 would therefore copy existing DNA methylation sites without creating new sites thus conserving DNA methylation patterns through mitotic cycles. This mechanism enables epigenetic memory and conservation of distinct DNA methylation profiles across cell lineages. De novo methyltransferases DNMT3A and DNMT3B can methylate completely unmethylated cytosines [36, 37] and DNMT3A could methylate cytosines in contexts other than CG [41]. De novo DNMTs are expressed at higher levels in embryonal stem cells and the brain [42]. Otherwise, de novo methylation is restricted and rare. The combination of restricted de novo methylation followed by maintenance methylation provides a mechanism for a transient event that triggers de novo methylation to be preserved for the long term through the automatic process of maintenance methylation by DNMT1. This provides a mechanism for establishing lineage specific DNA methylation profiles whereby

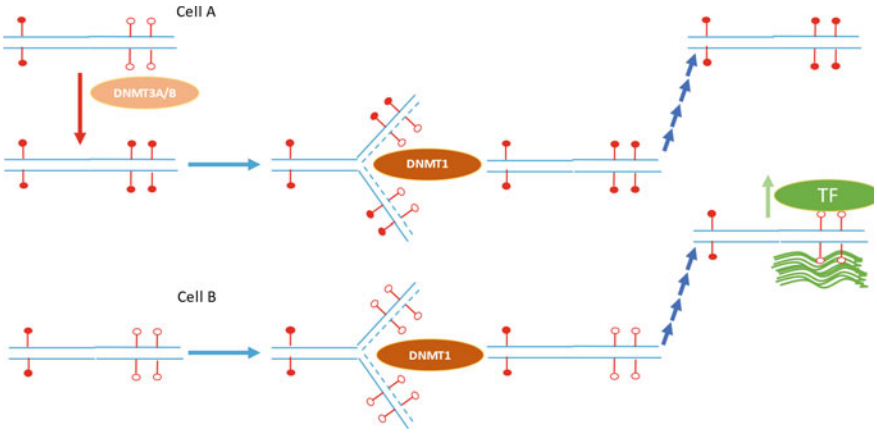

Fig. 1 Combination of De novo and maintenance DNA methylation, a mechanism for genomic "anticipation." A new methylation event at a point in development by de novo methyltransferase (DNMT3A or B) is perpetuated by maintenance methyltransferase (DNMT1) which faithfully copies the new methylation during DNA replication (replication fork is presented), the new pattern is maintained in the lineage derived from this cell but the lineage derived from another cell that escaped this de novo methylation even would maintain the ancestral methylation profile (open circles indicate CG positions, filled circles indicate methylation). However, although the lineages have different methylation both lineages don't express the gene because of a missing factor. Once this factor is induced later in development an expression differences (presented as the green lines) will be recognized between the two lineages

de novo methylation at different stages of development occurring in response to developmental-time specific signals are automatically propagated in the lineage by maintenance DNMT1. Similarly, it provides a mechanism for embedding and memorizing transient experiences and exposures and "anticipation" of future long term gene regulatory responses (Fig. 1).

The sculpting of lineage specific DNA methylation profiles during development involves demethylation as well as de novo methylation [21, 27, 43]. Similarly, responses to experiences and exposures involve demethylation as well as de novo methylation [44]. Once a methylated moiety on a cytosine in DNA is lost, this loss of methylation will be perpetuated in the downstream lineage of the founding cell since DNMT1 can only copy a methylated cytosine that exists on the template strand during DNA replication.

The methyl group on DNA is further modified by TET dioxygenases sequentially to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxycytosine [45, 46]. There are 3 TET genes in mammals [46]. It is unclear whether the oxidized methyl moieties serve as additional epigenetic marks or just as intermediates in DNA demethylation [47] as discussed below.

There are several mechanisms for demethylation which reflect the different roles that DNA methylation might be playing. DNA methylation could be lost through a "passive mechanism" which could involve a general absence of limitation of DNMT1

during DNA replication as is the case during early stages of embryonal development [48, 49] or a steric hindrance of DNA methylation during DNA synthesis by binding of a specific transcription factor to a specific DNA methylation target [50], leading to site specific demethylation. It is believed that binding of pioneer transcription factors to critical regulatory regions in DNA play an important role in site specific demethylation of regulatory regions such as enhancers [51]. Indeed, demethylated CG positions were utilized to impute transcription factor binding positions [52].

The oxidation of the methyl moiety in 5-methyl cytosine could bring about demethylation by either passive or active mechanisms. Oxidized methylated CGs are not copied readily by DNMT1 and therefore they would be lost at the next round of replication by passive hindrance of the DNA methylation reaction catalyzed by DNMT1 [53]. Oxidation of the methyl moiety can also trigger active demethylation. Bases with oxidized 5' methyl moieties target glycosylases such as TDG which remove the oxidized base, which is followed by repair of the "abasic" positions [45, 47]. This process is considered active since it doesn't require DNA replication.

However, notwithstanding the mechanism of demethylation any loss of a methylated site is thereafter perpetuated. Demethylation can happen on either strands or both. Once an unmethylated DNA strand is replicated the nascent stand would remain unmethylated as well since DNMT1 would not methylate it. A time limited signal that triggers demethylation is memorized in the genome by permanent loss of DNA methylation. Demethylation on one of two strands will result in differentially methylated daughter strands which will now be perpetuated in their two lineages creating potentially two different phenotypes if these methylation sites are strategically positioned. An event at a single early time point could thus have lasting effects on gene function or "anticipate" future effects on gene function as will be discussed below.

3 Functional Role of DNA Methylation: An Anticipatory Mechanism

The first characterized role of DNA methylation was in "restriction modification" in bacteria [54]. Different strains of bacteria harbour a sequence specific DNA methyltransferase and a restriction enzyme that cleaves the same sequence only when it is unmethylated [54]. An intruding exogenous phage which is unmethylated would be cleaved while the methylated host DNA would be protected. This ancient evolutionary role of DNA methylation as a defense mechanism and a cellular immune function reveals its first principles; DNA methylation functionally differentiates identical sequences, thus providing an additional layer of information unto DNA. In the case of restriction modification, the state of DNA methylation differentiates "self" from "other". Second, DNA methylation alters the interaction of proteins with DNA. DNA methylation modulates other genomic functions in bacteria such as mismatch repair, replication control and gene expression [55].

Transcription requires interaction between the transcription machinery and its cofactors and the DNA, which could be altered by DNA methylation at the binding targets of these factors [56]. The idea that DNA methylation alters transcription states arose from the inverse correlation observed between methylation of CG sites at 5′ regulatory regions of genes and steady state mRNA levels [57–61]. CG is the main dinucleotide sequence methylated in vertebrates and is the only methylated sequence context that could be copied as discussed above [38, 39]. The initial observations made using CG methylation sensitive restriction enzyme *HpaII* and its CG methylation insensitive isoschizomer *MspI* [58, 59] were replicated by whole genome bisulfite sequencing [43, 62–64]. This method uses chemical deamination of all unmethylated cytidines to uridines but leaves intact methylated cytidines, creating a sequence difference between methylated and unmethylated cytidines that is mapped using either Sanger or next generation sequencing [65, 66]. There is a genome-wide significant but not perfect inverse correlation between methylation states and steady state mRNA levels determined by RNAseq, a method that quantifies mRNA levels by global next generation sequencing. Promoters of most genes are unmethylated while the rest of the genome is heavily methylated creating a bimodal distribution of states of methylation [67]. The distribution of CG sequences in the genome appears to be bimodal as well with dense distribution of CGs in what was defined as CG islands and sparse distribution of CGs in the rest of the genome [63]. CG islands are mostly unmethylated and tend to concentrate in regulatory regions of housekeeping genes [68]. Regions with intermediate density of CG dinucleotide characterize differentially methylated, tissue-specific promoters [63, 69]. A comprehensive analysis of epigenetic marking during brain development revealed how loss/gain in DNA methylation corresponds with transitions in gene expression states and other epigenetic marks [43].

The inverse correlation between steady state mRNA levels and methylation and the absence of DNA methylation in all “house keeping” CG rich promoters and in all promoters that are actively transcribed suggests that DNA methylation in promoters and possibly other regulatory regions silences gene expression. A methylated moiety on cytosine predictably alters the interactions of proteins with a sequence that contains or is in vicinity of this cytosine [56]. Transcription factors that activate transcription were shown to be precluded from binding by DNA methylation in their binding targets, which results in silencing or downregulations of the transcripts activated by these factors [56, 70]. Alternatively, DNA methylation might also interfere with binding of transcriptional repressors, in these cases methylation of repressor target positions in DNA would possibly result in gene activation. DNA methylation at a target site could also potentially increase the affinity of a binding protein if a protein evolved to recognize the methylated moiety [71]. A family of proteins that are dedicated to methylated DNA (MBD) are found in many organisms [72]. These proteins could recruit other epigenetic modifiers that in turn silence transcriptional activity [73]. For example, the methylated DNA binding protein 2 (MeCP2) silences gene expression by recruiting the protein complex which contains the transcriptional repressor Sin3A and histone deacetylases (HDAC); histone deacetylation inactivates

chromatin and silences transcription [33]. However, it should be noted that methylated DNA binding proteins have multiple roles in regulation of gene expression and were also shown to activate certain genes by binding to unmethylated promoters [74] or by inhibiting methylation or by promoting DNA demethylation [75].

Although inverse correlations between promoter/enhancer methylation and steady state mRNA levels have been documented in numerous studies the genome wide correlation between transcription and methylation is imperfect [67]. Partially this has to do with the cellular heterogeneity of tissues and the difference between a digital binary signal which is generated in bisulfite sequencing and analog signal of mRNA counts. DNA methylation is binary signal, a site could be either methylated or unmethylated and therefore the fraction of methylation indicates the number of cells in the sample that have a methylated copy of the gene whereas the level of expression could reflect high expression of only few cells in the population or moderate expression in most of the cells. So, it is possible that a sample even of a cytologically homogenous population of cells will show high methylation indicating that most cells in the population are methylated and high expression that is derived from the small fraction of cells that are unmethylated. When we examined this question by isolating the copies of the gene that were physically engaged in transcription using antibodies targeting active transcriptional complexes that were physically interacting with the promoters we found that indeed this was the case. All promoters that were physically engaged in transcription in the sample were indeed fully unmethylated [67]. There was no case of a methylated promoter that was physically engaged in transcription [67].

In summary, DNA methylation as we understand from restriction modification systems in bacteria alters the interactions of proteins with DNA by modifying the chemical structure of the recognition sequence for these proteins. DNA methylation plays a similar role in higher organisms by altering binding of factors that regulate transcription. These effects could be either inhibitory as is the case with several restriction enzymes or transcription factors or. DNA methylation can therefore alter gene expression in both directions through altering the interactions of protein factors and DNA elements. Thus, although DNA methylation in promoters and enhancers silences gene expression, DNA methylation could potentially enhance gene expression if it is found in repressor sequences. Notably, DNA methylation in gene bodies is associated with active transcription in insects [76] as well as vertebrates [77, 78] probably though silencing cryptic promoters in the gene body [79]. The evolution of DNA methylation and its further modifications by oxidation confers enormous plasticity to the genome as identical genomes could express numerous functions through different combinations of DNA methylation profiles in different cells and different tissues. It creates enormous potential for heterogeneity at the cellular, tissue and individual levels. The pattern of methylation as we shall see below is not a mere blueprint of transcription steady state, but it anticipates future events in developmental trajectories and in response to future exposures and experiences. At the basal level this is conserved in the DNA sequence as the evolutionary distribution of CGs in the genome anticipates the profiles of DNA methylation that will appear at different times of development, methylation profiles anticipate future life trajectories while

experience mediated DNA methylation changes anticipate future experiences which will be discussed below.

4 DNA Methylation as an Anticipatory Gene Expression Regulator

DNA methylation is not a mirror image of steady state mRNA levels. Thus, a simple correlation of steady state mRNA and DNA methylation as is routinely presented in the literature doesn't reflect the complexity and sophistication of DNA methylation role in setting up gene expression programs. Although promoters that are engaged in transcription are invariably unmethylated as discussed above, many unmethylated promoters are silent [67] (Fig. 1). Living systems are responsive and react to changing developmental, internal and external signals. Thus, steady state levels of mRNAs do not reflect the responsive organism physiology. It should be noted that many important genes are activated only in response to specific triggers, for example glucocorticoid hormone regulated genes [80] and interferon genes induced by viral infection [81] and various other genes which are only activated in the appropriate physiological context. These genes are activated by transcription factors that are downstream mediators of external and internal signals [82]. DNA demethylation is a necessary but insufficient condition for transcription. The demethylation of a promoter poises it for expression only in the right context when other transcriptional factors are activated by other signals and interact with the unmethylated promoters (Fig. 1). Demethylation is setting the stage for future activation when the right developmental, physiological, or environmental conditions exist. A classic example is the demethylation of glucocorticoid response elements (GRE) in the enhancer of the Tyrosine Aminotransferase gene *tat* by exposure to glucocorticoids which remains demethylated after hormone withdrawal, doesn't affect the basal state of expression but results in a stronger transcriptional activation in response to subsequent exposure to glucocorticoids [83]. Demethylation of the GRE serves as a genomic memory of the first encounter with the hormone and anticipates future exposures to the hormone [83]. DNA demethylation thus anticipates the future responsivity of the gene and sets the stage for future responses. Differential methylation might therefore have little impact on steady state levels of expression but a dramatic effect on future physiological responses. Similar mechanisms might be operating in setting up future developmental trajectories of gene expression. Demethylation can anticipate activation of a gene once additional transcription factors are activated by subsequent demethylation later in development. Different sequences of methylation/demethylation downstream of a single demethylation event would result in different cellular lineages derived from one demethylated ancestral cell.

5 DNA Methylation Alterations by Early Life Experience Anticipating Responses to Future Exposures and Experiences

While developmental trajectories of DNA methylation are anticipated by evolutionary history of the DNA sequence and are predictable and consistent across individuals at different times in development, experiences and exposures including social experiences also alter DNA methylation trajectories particularly early in life which affect phenotypes later in life.

Mouse maternal diets during pregnancy alter the methylation of a transposable element inserted in the agouti gene anticipating differences in coat color and obesity, diabetes and risk for tumors that emerge later in life [84–87]. Early life responses in DNA methylation to exposures are not limited to chemicals or nutrients but are also triggered by the social environments and behavioral experiences. The intensity of early life maternal care in the rat affects the stress responsiveness of the offspring; offspring of high maternal care rats show reduced stress reactivity later in life compared to offspring of low maternal care [88]. The differences in stress response could be explained by differences in expression of glucocorticoid receptor in the hippocampus which emerge early after birth in response to maternal care and remain into adulthood [88]. The glucocorticoid receptor in the hippocampus plays an important role in feedback inhibition in the HPA axis, which controls the extent of the stress response [89]. The differences in expression of the glucocorticoid receptor gene (*nr3c1*) are associated with differences in DNA methylation and histone acetylation in exon 1_7 promoter of the gene [90]. These differences in offspring stress responsivity are triggered by maternal behavior rather than a genetic difference since cross fostering of the pups reveals that it is the caring mother rather than the biological mother that affects offspring stress responsiveness and glucocorticoid receptor expression [91].

A pathway linking maternal care and changes in DNA methylation was proposed, maternal care triggers a serotoninergic signaling in the hippocampus which activates the transcription factor NGFIA, binding of the transcription factor to the promoter recruits histone acetylase CBP and methylated DNA binding protein Mbd2 to the promoter which in turn alter acetylation and methylation [92, 93]. The epigenetic and gene expression differences remain into adulthood. Thus, epigenetic and gene expression alterations in response to maternal care anticipate future social behavior.

The changes in DNA methylation and gene expression in response to maternal behavior are not limited to the stress response and affect broader methylation and transcription profiles [44, 94]. These responses are evolutionary conserved and are seen as well in humans; child abuse is associated with differences in expression and methylation of the glucocorticoid receptor in the hippocampus [95] as well as broad changes in DNA methylation in syntenic genomic loci [44]. Other studies have shown that early life adversities such as exposure to abusive caretaker are associated with changes in DNA methylation in *Bdnf* [96], early life stress with methylation change in arginine vasopressin Avp [97], prenatal stress with alterations in Glycoprotein

M6A (*Gpm6a*) [98] and maternal separation with strain specific changes in DNA methylation of *Nr3C1*, *Avp* and *Nrda4* [99]. These early life alterations of DNA methylation remain into adulthood and are associated with later behavioral changes.

An interesting practical application of anticipatory epigenetics programming is using early life DNA methylation changes as predictors for risks later in life. Mice that have been genetically depleted of one copy of the glucocorticoid receptor gene exhibit alterations in DNA methylation in placenta, an accessible tissue at birth. Several of the changes in DNA methylation at birth predict risk for anxiety like behaviors in the adult mice later in life [100].

Studies in the rhesus macaque which compared maternal, and nursery reared monkeys revealed differences in multiple behaviors as well as differences in DNA methylation in brain and the immune system in adult monkeys [101]. These differences in DNA methylation emerge early after birth and maternal separation but they are dynamic [102]. DNA methylation profiles evolve through early life with large changes around weaning in all animals, however the trajectory of evolution of DNA methylation profiles is different between maternally reared and nursery reared animals [102]. The changes in methylation induced by loss of the mother early in life are not fixed but trigger a dynamic "cascade" of alterations in DNA methylation that evolve developmentally. Early alterations in DNA methylation in response to maternal separation activate a sequence of later changes in DNA methylation. Although there is no evidence that all these changes in DNA methylation affect the phenotype, it is speculated that some of these differences in the developmental trajectories of DNA methylation are associated with behavioral and physiological changes in adulthood. The initial social environment that sets in motion the shift in the trajectories of DNA methylation is not "deterministic" but "anticipatory." Downstream exposures might modulate the direction of these trajectories.

The study of dynamic epigenetics in living humans is extremely challenging especially when it pertains to behavior. DNA methylation and other epigenetic processes are highly tissue specific and the relevant tissue for behavior is the brain which is inaccessible in living humans at least for analyzing DNA methylation at a base specific resolution. The only accessible tissues are blood and saliva whose relevance to brain function is uncertain. However, since stress is known to have systemic effects on both the immune and metabolic systems, investigation of changes in DNA methylation in blood provide us with a picture of the impact of early life events on immune and inflammatory processes which are known to play a critical role in chronic disease and behavioral states. Another main challenge is determining causation between the behavioral environment and DNA methylation alterations and between changes in DNA methylation and phenotypic alterations. Human disasters offer an opportunity for a quasi-experimental study design. The Quebec ice storm of 1998 that affected the Quebec electric grid in the dead of winter offered such an opportunity. Children who were born around the storm date were followed up into adolescence and the objective stress of their mothers was assessed [103]. The children developed increased incidence of autism, sugar intolerance and asthma that correlated with antenatal maternal stress [104–108]. Genome wide DNA methylation analysis in T cells from the 15-year adolescents revealed broad variations in DNA methylation that correlated with

the level of antenatal maternal stress [109]. Mediation analysis suggested that these changes in DNA methylation mediated the impact of maternal stress on cytokines [110], and BMI [111]. Although the DNA methylation profile of these adolescents at birth was not measured, these data are consistent with the hypothesis that maternal stress triggered changes in DNA methylation at birth that anticipated later changes in metabolic, immune, and behavioral systems. The variance in the response is consistent with the idea that these effects are anticipatory but not deterministic and that later events can shape the DNA methylation and behavioral responses to the anticipatory changes early in life.

6 The Epigenetic Response to Early Life Environment: An Anticipatory Adaptive Mechanism

A large body of data suggests that early life social and physical environment can determine the set point for lifelong trajectories of physical and mental health. For example, adverse childhood events (ACE) can have adverse effects on adult health [112] and nutritional restriction during pregnancy and reduced birthweight is associated with higher risk for chronic disease in adulthood [113]. The effects of early life adversity are not limited to disruption of physical health but include mental health and behavior as well as discussed here. The idea that early life conditions determine the emergence of chronic disease is embodied in the DOHAD (developmental origins of health and disease) concept [114]. This is consistent with an anticipatory mechanism whereby the biological responses to early life experiences anticipate downstream events that would emerge in adulthood. This is anticipatory but not deterministic since the effects are heterogeneous and other events later in life could modulate or possibly overturn the effects of early life exposure. DNA methylation and epigenetic mechanisms were postulated to be mediating and embedding in the genome the effect of early life exposures [115].

What are the mechanisms that mediate between early life exposures and epigenetic programming and how are they playing their anticipatory role? What is the evolutionary advantage of these mechanisms that selected and preserved them? There are many physiological signaling pathways that sense nutritional states and stress that are operative in the developing embryo and later in the developing child. The stress hormone glucocorticoid is a good example for such a mechanism. The glucocorticoid receptor is a nuclear factor that has been known to alter epigenetic states [83, 116–118]. The glucocorticoid receptor also integrates the control of immune/inflammatory, brain/behavior and metabolic responses [119, 120]. It is also expressed in numerous tissues including sperm and can integrate epigenetic responses across multiple tissues and multiple physiological systems [121]. Other similarly nodal-hormones are oxytocin and insulin. These hormones respond to external and internal signals and trigger a variety of physiological responses. By epigenetic reprogramming the transient signals which normally elicit a transient

physiological response could be embedded in the genome as DNA methylation alterations and become long-lasting genomic memories of these transient signals. What makes DNA methylation a truly anticipatory mechanism is that demethylation by itself is not always sufficient to activate the response. It requires other factors that might be activated later in life or in response to an exogenous signal. This could explain how changes in DNA methylation early in life will have an impact on the phenotype only later in life as postulated by the DOHAD hypothesis. The DNA methylation alterations anticipate these events but do not determine the response; the response is dependent on the occurrence of the anticipated signals. Also, the impact of the response when it occurs would depend on the future environment and the level of similarity to the environment that triggered the epigenetic reprogramming in the beginning.

A good example is the response of the proximal regulator of glucocorticoid receptor activity FK506 binding protein 5 (*FKBP5)*. *FKBP5* binds the glucocorticoid receptor and prevents it from localizing to the nucleus serving as a negative regulator of glucocorticoid response [122]. The gene encoding FKBP5 has an intronic glucocorticoid response element (GRE) that binds hormone activated glucocorticoid receptor inducing expression of *FKBP5*, increasing its levels, and suppressing the glucocorticoid response [123]. FKBP5 serves as a negative feedback loop for controlling and tampering glucocorticoid responses whereby increase in glucocorticoids will activate *FKBP5*, suppressing the glucocorticoid response [123] (Fig. 2).

Early life adversity is associated with demethylation of an intronic GRE of the *FKBP5* gene [124]. However, this epigenetic programming of the *FKBP5* gene will only have a physiological effect when there is a surge in glucocorticoids in response to a strong stressor later in life such as a traumatic experience [124]. *FKBP5* is programmed in anticipation of strong stress exposure by the cues of early life environment which anticipate a stressful life, but its physiological and phenotypic impact is dependent on the presence of a strong stressor later in life. People who were exposed to child adversity early in life and have demethylated *FKBP5* will have a higher risk for developing PTSD later in life when exposed to a traumatic experience, but no significant impact of this demethylation will be observed under non stressful conditions [124]. It is important to note that a genetic difference in the sequence of the regulatory element of *FKBP5* increases the probability of demethylation of *FKBP5* by early life adversity, a gene by environment interaction [124]. Thus, the genetic difference in *FKBP5* anticipates demethylation which will only happen if there is early life adversity which results in a surge of glucocorticoids. The demethylated state of *FKBP5* anticipates a muted response to glucocorticoid surge which occurs if there is an exposure to trauma. Interestingly, differential methylation of *FKBP5* was reported in second generation of holocaust survivors [125].

This demethylation of *FKBP5* by early life stress could be mimicked in a progenitor neuronal cells culture by treatment with the synthetic glucocorticoid dexamethasone [126]. The demethylation of *FKBP5* would have no effect on the basal state of the neurons. However, if the differentiated neurons which recapitulate the status of neurons in an adult brain are exposed to a second round of glucocorticoids, *FKBP5* will be elevated and mute the response to glucocorticoids [126]. This study provides

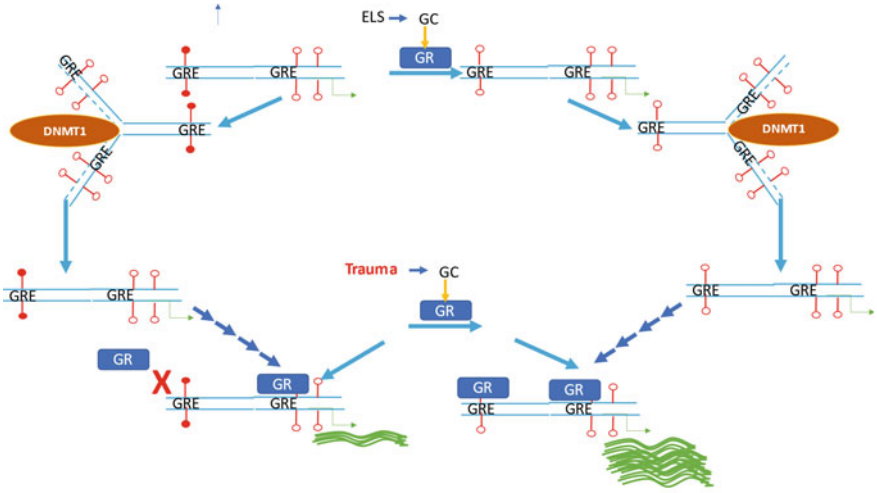

Fig. 2 DNA demethylation of a GRE element in FKBP5 during childhood anticipates future responsivity of the gene to trauma. Early life stress increases glucocorticoid levels (GC), binding of GC to the glucocorticoid receptor (GR) activates it and causes demethylation of a GRE (glucocorticoid responsive element) in the 7th intron (open circles indicate CG positions, filled circles indicate methylation). After stress is resolved there is no impact on gene expression. The new pattern is maintained by maintenance DNA methyltransferase (DNMT) for multiple generations (indicated by the sequence of arrows). Children who were no exposed to stress (left scheme) will not undergo demethylation. In absence of trauma there is no difference in expression. However, upon exposure to trauma, there will be a robust response to the surge of glucocorticoid hormone in people who have the GRE site unmethylated (indicated by the green lines indicating mRNA synthesis) while people who have the GRE methylated would have a tampered response to the trauma

a mechanism for long lasting epigenetic reprogramming by early life stress and supports a critical role for glucocorticoid receptor in mediating the impact of early life experiences on epigenetic programming. Depletion of one copy of the glucocorticoid receptor in mice results in sex specific alterations in DNA methylation in the placenta supporting the critical role of glucocorticoid receptor in shaping DNA methylation during embryonal development [100].

It is postulated that such a system evolved to enable a stable genome to respond to dynamic environmental conditions without having to resort to natural selection as the only mechanism for long lasting adaptation. The early life environments provide the developing organism with cues to the anticipated life-long environment. To adapt the genome to the anticipated environment, gene expression programs should be modulated to increase fitness in the anticipated environment. For example, nutritional restriction signals an expected impoverished life. This requires adapting eating habits towards binging and metabolic balance that is directed to storage of energy in fat. However, if the anticipated environment differs from the observed environment, this adaptation can result in maladaptation and disease. Binging and fat accumulation are detrimental under the conditions of ample calories availability. A similar hypothesis

could be constructed around the immune/inflammatory and brain/behavior systems. For example, early life stress anticipates a challenging social environment later in life which requires heightened stress responses. But these might become maladaptive under normal social conditions later in life if there is a misfit between the anticipated and observed social environment. Early life adversity signals future social, metabolic, and immune challenges as these three environments are evolutionary interrelated.

7 Summary

The emergence of cell type specific DNA methylation patterns during development and in response to experiences and exposure early in life is a multitiered anticipatory mechanism.

a. The genomic sequence anticipates the emergence of distinct methylation profiles at different times during development but the methylation profile that emerges is different in different tissues and might be influenced by stochastic or physiological and environmental signals during development. Although a cell type specific methylation profile is anticipated, the methylation patten that emerges could vary by downstream conditions and contexts.
b. Changes in DNA methylation anticipate future changes in gene expression but are not deterministic as these changes require additional factors that would only occur under certain anticipated environmental triggers or developmental conditions.
c. Environmental exposures early in life anticipate life long environmental conditions and alter related DNA methylation profiles to adapt the genomic program to these anticipated environments. The changes in DNA methylation triggered by early life environments are dynamic. Initial epigenetic alterations trigger and anticipate a downstream cascade of epigenetic alterations. The changes in DNA methylation vary across individuals and could be affected by genetics and diversity of external and internal signals.
d. These anticipatory DNA methylation profiles express their effects later in life in response to later developmental and exogenous environmental and internal physiological triggers. The extent to which the original anticipatory DNA methylation profiles impact the phenotype would vary by external and internal conditions later in life. A misfit between anticipated and observed environments could result in maladaptation and either physical or mental disorders and is consistent with the hypothesis of developmental origin of health and disease. An anticipatory mechanism of gene function enables a fixed genome to function in a dynamic environment.

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The Genetics and Epigenetics of Anticipatory Adaptation

Bernhard Horsthemke

Abstract According to Darwin, a species adapts to the environment by variation and natural selection. During their lifetime, organisms adapt to changing environments by phenotypic plasticity. Based on an internal predictive model, organisms can anticipate future environments and preadapt accordingly. Alternatively, they may hedge their bets by random phenotypic variation. Predictive models, phenotypic plasticity and bet hedging are genetic traits. While gene-regulatory networks have a primary role in bringing the phenotype into being, chromatin modifications, which are often referred to as epigenetic changes, affect the local kinetics of gene expression. They stabilize cellular states and are rarely transmitted to offspring.

Keywords Adaptation · Anticipation · Genetics · Epigenetics · Phenotypic · Plasticity · Bet hedging

1 Introduction

"The future interests me—I'm going to spend the rest of my life there", said Mark Twain. This is probably true for all of us. Humans can make decisions based on a cognitive model of the future and act in appropriate ways. Non-human animals also can show anticipatory behaviour, although in a more narrow domain [1]. Being able to adapt or even preadapt to changing environments is important for survival and reproductive success of all organisms. Since this ability increases the fitness of an organism, it has been positively selected during evolution and is encoded in the genome. Nevertheless, different species use different strategies for anticipating and adapting to changing environments. The strategy also depends on the type of the environmental change, which can be classified by the relative length of an environmental period and the pattern of the environmental change.

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2 Types of Environmental Change

2.1 The Relative Length of an Environmental Period

The environmental period can be shorter, equal too, or longer than the lifespan of an organism. If the environmental period is much shorter than the lifespan of the organism, the organism will face many environmental changes during its lifetime. If the environmental change is equal to its lifetime, the organism will live in a constant environment, but its offspring will encounter a different environment. If the environmental period is much longer than its lifetime, several generations will live in a constant environment, before a generation will encounter a new environment. In this article I will focus on the first scenario.

2.2 The Patterns of Environmental Changes

The patterns of an environmental change can be classified into regular, irregular and rare changes. Regular changes are changes that occur repeatedly at a fixed frequency. An example are the seasons of a year, which depend on the rotation of the earth around the sun. This is in contrast to environmental events which occur at irregular intervals (e.g. seed-rich years) or are extremely rare (e.g. the eruption of a volcano or the impact of a meteorite).

3 Anticipation of a Future Environment

While adapting to a new environment is good for an organism and a population, anticipating a future environment is even better. Here I will focus on birds and mammals. Some species have developed a way to respond to the different seasons of the year. In anticipation of the winter, many birds migrate to warmer countries, other animals begin hibernation, and the Arctic fox (*Vulpes lagopus*) grows a white fur. The seasonal rhythm of these animals is based on an endogenous timer (circannual clock), which is synchronized by the seasonal changes in photoperiod [2] and probably influenced by other environmental information also. The cyclic behavioural and physiological changes of these animals evolved in response to cyclic and predictable changes of the environment. For a great part, this response is encoded in the genome; breeding experiments in migrating birds, for example, have shown that the direction and duration of the flight are genetic traits [3]. Clock-based phenotypic changes, however, carry some risk. If the start of the snowfall, for example, is delayed in a warm year, the white Arctic fox will be maladapted until the first snowfall.

Anticipation of a sporadic environmental event is also possible, if such an event occurs more than once during the lifetime of an organism and if there are harbingers of

the future environment. A good example are chipmunks (*Tamias*), squirrels (*Sciurus carolinensis*) and dormice (*Glis glis*), which adjust their reproductive activity to match juvenile weaning with peak seed availability of masting beech trees, which are essential for their survival [4–6]. Females have more offspring in spring and summer, if beech trees produce large amounts of seeds in the following autumn. It is unknown what cues these animals use to predict mast seeding, but studies have suggested that visual or chemical stimuli, possibly linked to reproductive plant structures (buds, flowers, pollen cones), can trigger onset of reproduction [4]; these structures are consumed by chipmunks, squirrels and dormice and are present in advance of and correlated with the size of the forthcoming seed crop [4]. Although the mechanistic link between the consumption of these structures and fertility is still unknown, anticipatory reproduction of seed predators is probably a genetically determined trait. Remarkably, dormice resume hibernation, if they anticipate poor beech seed availability later in the year and therefore decide not to reproduce in spring [6].

If the future is unpredictable, a risk spreading strategy (bet hedging) can be advantageous. A good example are Norwegian house sparrows, which lay eggs of varying sizes in the same clutch. The difference in egg volume can vary by 50%. While increased egg size reduces offspring mortality in early life, especially under heavy precipitation, decreased egg size does so at higher temperatures [7]. The variation in egg size ensures that there are always some eggs of optimal size for any weather condition.

4 The Genetic Basis of Adaptation and Anticipation

4.1 Phenotypic Plasticity

Although all cells of a multicellular organism have the same genotype, different cell types differ in structure and function, i.e., in their phenotype. Differentiation is based on cell-autonomous processes (e.g., toggle switches involving transcription factor feedback loops) and signals from neighbouring cells. Likewise, genetically identical organisms express different phenotypes in response to different environments. This phenomenon is called phenotypic plasticity (Fig. 1a). The range of phenotypes produced by a particular genotype in different environments is determined by the norm of reaction, a term first introduced by Woltereck in 1909 [8] (Fig. 2). The norm of reaction and the degree of plasticity are a property of the genotype. Phenotypic plasticity is based on environmentally responsive genes and loci responsible for variation in reaction norms [9].

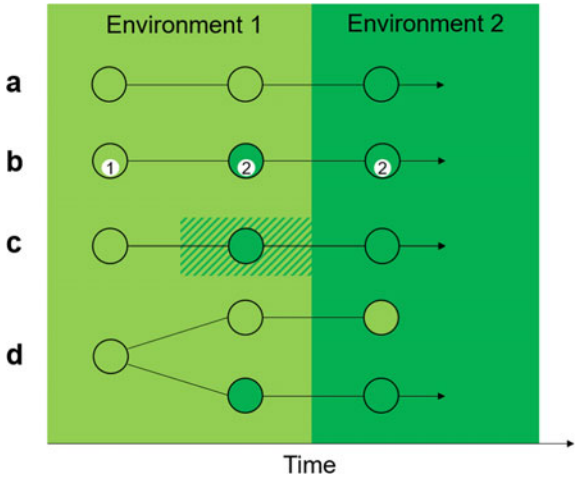

Fig. 1 Anticipation of future environments. Two successive environments are shown in light and dark green. Circles represent an organism at different stages of its life, the arrow its life history. Adaptation of the organism is indicated by a circle with a colour that matches the environment. **a** Phenotypic plasticity, **b** preadaptation based on an endogenous clock (small white circle), **c** preadaptation based on an environmental cue linked to environment 2 (striped colours), **d** bet hedging. Owing to random variation, genetically identical organisms in a population can have two different phenotypes in environment 1, one of which is perfectly adapted to environment 2

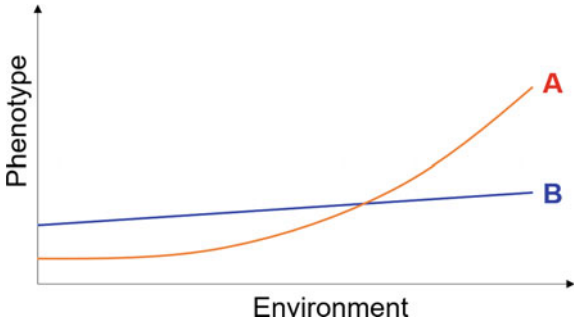

Fig. 2 The norm of reaction determines how environmental variation (horizontal axis) contributes to phenotypic variation (vertical axis). Different genotypes (A and B) have different norms of reaction (red and blue lines). A is more plastic than B

Phenotypic plasticity can be a simple reaction of the organism in response to some basic environmental factor such as temperature or nutrient supply (physiological plasticity). In these cases, phenotypes are often reversible. At critical developmental windows, however, the environment can change developmental trajectories and cause irreversible phenotypes (developmental plasticity). Maternal nutrition and stress levels, for example, can affect the developing embryo and have life-long consequences. A good example is the Dutch famine of 1944–1945. Epidemiologic studies have shown that severe undernutrition of pregnant women in the first trimester increased the risk of their offspring to develop cardiovascular disease in later life. Probably, the children who experienced food restriction in utero were maladapted to

the food-rich environment after birth and therefore developed chronic disease (see also below).

Phenotypic plasticity is anticipatory, when an organism does not only react to the current environment but also to predictions about the future environment as made by an internal predictive model [10] (Fig. 3). Such a model can, for example, involve an endogenous timer (Fig. 1b; see the discussion on seasonal rhythms in animals) or the recognition of a link between successive environmental events (Fig. 1c; see the discussion on the reproductive activity of squirrels in a year with mast seeding of beech trees). An internal predictive model distinguishes complex biological systems from simple machine-like systems [10]. Different species have different predictive models, which indicates that the basic components of these models are encoded in the genome.

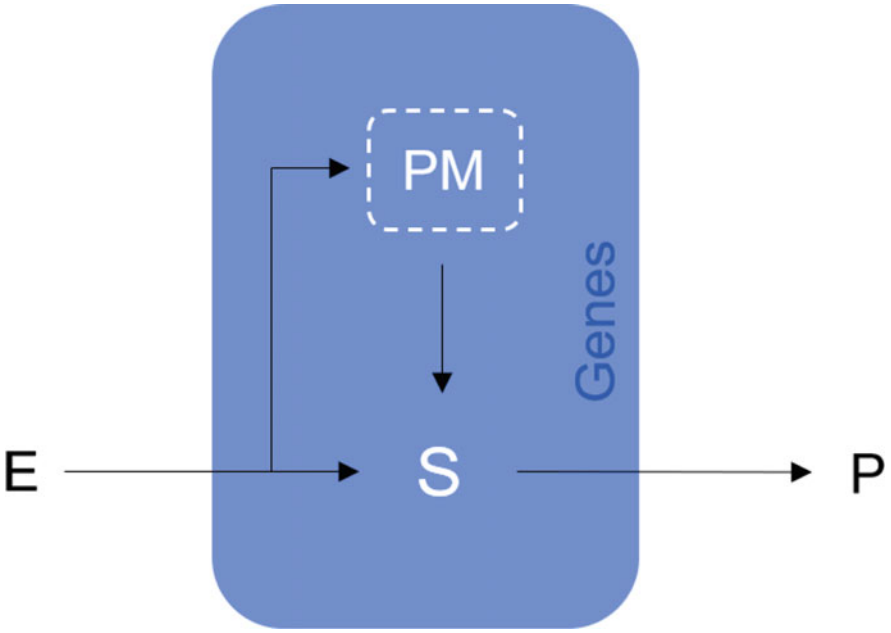

Fig. 3 A complex biological system with an internal predictive model. The system (S) receives input from the environment (E) and an internal predictive model (PM) and reacts with phenotype P [10]. Thus, the current state of the system does not only depend on the past states of the system but also on the possible future states. The predictive model and the norm of reaction are genetic traits and species-specific

4.2 Bet Hedging

Genotypically identical cells and organisms can not only develop different phenotypes in response to different environments, but even in the same environment. This is because of stochastic fluctuations in gene expression and developmental noise. Although random phenotypic variation may appear to be a costly error of nature, the ability to generate random phenotypes can actually be encoded in the genome and be adaptive [11, 12]. Some of the randomly occurring phenotypes may not be optimal for the current environment, but may by chance be perfectly adapted to a new environment and then have a selective advantage (Fig. 1d). This form of risk spreading strategy (see e.g. the egg size of the Norwegian house sparrow) has been called "diversified bet hedging" [13] or "adaptive coin flipping" [11]. Some evolutionary biologists distinguish between these two types of bet hedging, but for the sake of simplicity I lump them together here.

Random phenotypic variation in the absence of genetic and environmental variation can occur, because—from a physicochemical perspective—gene-regulatory networks are complex non-linear dynamic systems with multiple attractors [14]. As argued by Huang [15], Waddington's metaphoric "epigenetic landscape" represents the quasi-potential function of the global network dynamics (Fig. 4). The dynamic system is based on gene-regulatory networks, and owing to gene expression noise, the system can occupy different states. In going beyond Neo-Darwinism, Huang has suggested that randomly occupied phenotypic states can be subject to natural selection [15]. Similarly, Feinberg and Irizarry [16] have suggested that stochastic epigenetic variation (which in my opinion is transcriptional variation reflected by "epigenetic" variation) can be a driving force of development, evolutionary adaptation and disease.

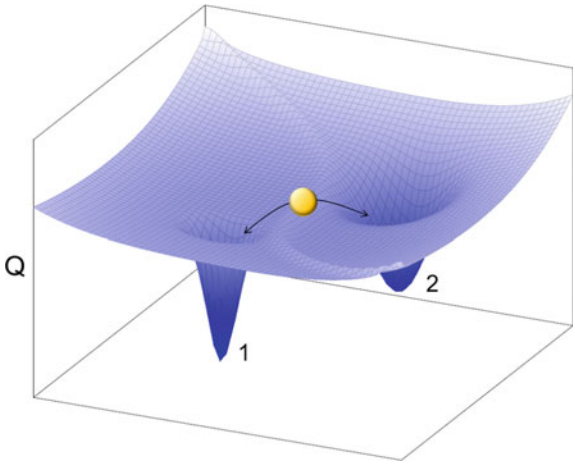

Fig. 4 Phase space of a dynamic system with two attractors. An attractor is defined as a state towards which a system tends to evolve. The two attractors of the system shown here are labeled 1 and 2. As indicated by the yellow ball and the arrows, the system can move towards attractor 1 or 2. Q; quasi-potential function of the global network dynamics

5 The Role of Epigenetics

It is often claimed that development involves an epigenetic program, which is influenced by the environment. It has even been suggested that pregnant females program their offspring and subsequent generations in anticipation of the future environment. However, if the environment remains the same, "fetal programming" is not really anticipatory, and if the environment changes, the offspring will be maladapted.

The term "epigenetics" was coined by C. H. Waddington, who defined epigenetics as "the branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being" [17]. Unfortunately, the term has taken on multiple meanings and is nowadays used to describe many different phenomena. With regard to molecular epigenetics I prefer the definition by A. Bird, who defined epigenetics as "the structural adaptation of chromosomal regions [by histone and DNA modification] so as to register, signal or perpetuate altered activity states" [18]. Good examples are genomic imprinting and X-chromosome inactivation, in which the repressed state of genes is maintained by DNA methylation. Other researchers subsume miRNA and other RNAs under the umbrella of epigenetics, but RNA is a diffusible molecule and carries DNA-based sequence information, i.e. this is a completely different story. For clarity, I try to avoid the term "epigenetic" here, but will use the terms "chromatin modifications" and "RNA". Being a DNA methylation researcher, I will focus on this type of chromatin modification.

A widespread misconception of epigenetics is that chromatin modifications constitute a distinct layer of gene regulation, that they can be directly modified by the environment and that environmentally induced changes are heritable. This view ignores basic molecular facts: (1) Chromatin modifications are an integral part of transcriptional regulation. In fact, transcription shapes the genome-wide DNA methylation and histone acetylation patterns [19, 20] (see also below). This does not exclude the rare occurrence of epimutations, which can affect the local kinetics of gene expression and cause disease [21]. (2) Chromatin modifications are not directly affected by environmental factors. Although it has been reported that DNA methylation of an IAP retrotransposon within the murine *agouti viable yellow* (A^{vy}) locus is increased after maternal dietary supplementation with folic acid [22], it is possible "that the increase in DNA methylation at the A^{vy} IAP is a secondary effect caused by downregulation of *agouti* transcription after methyl donor supplementation" [23]. (3) It is true that DNA methylation patterns are cell-heritable, i.e., copied from the parental DNA strands onto the daughter strands by the DNA methyltransferase DNMT1, but at least in mammals they are erased between generations (see paragraph on transgenerational epigenetic inheritance).

6 The Role of Chromatin Modification in the Development of the Phenotype

Changes in DNA methylation observed after exposure of a developing organism to environmental factors are often referred to as "epigenetic programming". Heijmans et al. [24], for example, have reported abnormal DNA methylation patterns in peripheral blood of individuals born to pregnant women in the Dutch hunger winter (see above). I do not think that this reflects epigenetic programming, because there is no epigenetic program, and without a program, there can be no programming. According to Bestor et al., "the available data do not support the existence of a biochemical system that regulates embryogenesis by programmed methylation and demethylation of regulatory sequences.… Dynamic gene activation and repression during development are controlled by conserved protein- and RNA-based pathways that are largely common to both methylating and non-methylating organisms." [25]. How do organism develop? By self-organisation, a process that involves gene-regulatory networks, feedback interactions between mechanical and biochemical factors as well as cues from neighbouring cells (for a review see [26]).

There is no doubt that severe maternal over- and undernutrition as well as stress can affect the developing embryo. This effect is mediated by signaling cascades, which activate or repress transcription factors (TFs). Pioneer transcription factors recruit chromatin modifying enzymes to "open" or "close" the chromatin at specific sites, thus allowing or preventing other transcription factors to bind and regulate their target genes [27]. "Although chromatin regulators are critical partners for TFs, they play a secondary role in the definition of cell fates. Rather, a primary function of chromatin during development is to reinforce or stabilize these lineages and cell fates" [28]. The ensuing altered patterns of cellular differentiation and proliferation result in the different cellular composition, structure and function of tissues and organs as well as hormonal and metabolic setpoints, which persist into adulthood. Altered DNA methylation patterns reflect the disease state or the altered cellular composition of tissues; they are a consequence rather than a cause of disease. Since each cell type has a characteristic pattern of DNA methylation, cell mixture distribution is a major confounder of DNA methylation studies, and so is genetic variation, especially in human case-control studies [29]. These confounders have been ignored in many studies.

7 Adaptation and Genetic Assimilation

After having looked at environmental changes that occur more than once during the life of an organism, let's now assume that an environmental change occurs once in the life of an organism and that the new environment then persists for many generations. In this case, the organism reacts with phenotypic plasticity to adapt to the new environment, and so do its offspring. At first glance, the presence of the

same phenotype in parents and offspring looks as if an environmentally induced trait had been transmitted from parent to offspring, possibly in order to give the offspring a selective advantage in the new environment. If chromatin modifications were measured, similar patterns would be found in parents and offspring, which might be interpreted as the result of transgenerational epigenetic inheritance. However, what is inherited are (1) the genes underlying the norm of reaction and (2) the environment. Generations sharing the same genes and environment will develop a similar phenotype, with chromatin modifications being established anew in each generation [30]. If the environment returned to its previous state during the life of one of the next generations, this generation would respond by exhibiting the original phenotype.

The persistence of the new environment for many generations may eventually lead to genetic assimilation as first described by Waddington. In 1953 he observed that after applying a heat shock to pupae of *D. melanogaster*, some flies developed crossveinless wings [31]. After selective breeding for this phenotype, the frequency of this heat-inducible phenotype increased in the fly population from generation to generation. After 14 generations, the crossveinless phenotype occurred even without a heat shock. Waddington concluded that "the crossveinless character, originally a typical 'acquired character', has become incorporated into the genetic make up of the selected races", that this process "depends on the tendency of selection not merely to increase the frequency of any favorable character, but also to stabilise its development", and that "the genetic basis of the assimilated crossveinless character is polygenic" [31]. Genetic assimilation is by no means Lamarckian or epigenetic inheritance; it is based on the exposure of cryptic genetic variants that are present in the population, or—more rarely—de novo mutations.

8 Transgenerational Epigenetic Inheritance

In contrast to plants, chromatin modifications that reflect the state of somatic cells in mammals adapted to their environment are rarely transmitted to the next generation. This is due to two reasons. (1) In mammals the germline is separated from somatic cell lineages during early embryogenesis (Weismann barrier [32]), and we do not know of any mechanism by which somatic cells could impose their chromatin state onto the genome of germ cells. (2) During early embryogenesis the genome undergoes two waves of global DNA de- and re-methylation, one shortly after fertilization and one during the development of the germ line. In the literature, these two events are often called "epigenetic reprogramming", but, as explained above, there is no program and hence no reprogramming. The changes in DNA methylation patterns are initiated by transcription factors, and their primary role is obvious from the Nobel prize winning induction of pluripotent stem cells by the overexpression of Oct3/4, Sox2, Klf4 and c-Myc [33]. This does not exclude that as a result of a rare de- or re-methylation error, a local DNA methylation pattern can occasionally survive these processes [34]. However, these rare events are neither anticipatory nor adaptive.

9 Anticipatory Adaptation in Humans

In contrast to other animals, humans can rapidly adapt to new and even extreme environments. Our species can live in all climate zones of the earth and even on the moon. This extraordinary plasticity is not based on our physiology, but on our brains and our ability to use tools, to speak and to collaborate with others. We conceive and construct an artificial environment around us to protect us against the real environment: clothes, space suits, homes, etc. People living in the North do not migrate to the South because of an endogenous clock, but because they enjoy having sunny holidays, and in anticipation of the burning sunbeams they take sunscreen with them. With our brains (and nowadays with our computers and artificial intelligence), we can analyse the past and predict the future, for example with regard to climate in the next decades. Unfortunately, other properties of our big brains often prevent us from living according to these predictions, but this is another story. Most importantly, we transmit our knowledge, culture and self-constructed ecological niche to the next generation. This is much more efficient than transgenerational epigenetic inheritance could ever be.

10 Summary and Conclusions

Organisms adapt to new environments, either by phenotypic plasticity or by random phenotypic variation (bet hedging). They can anticipate future environments that occur at regular or irregular intervals during their lifetime and preadapt to them by phenotypic plasticity, if they have an endogenous timer or can sense harbingers of the future environment. This form of anticipatory adaptation is based on an internal predictive model and specific for the new environment. Bet hedging is a non-specific risk-spreading strategy, which is beneficial in unexpected environmental situations. Predictive models, phenotypic plasticity and bet hedging are genetic traits. While gene-regulatory networks have a primary role in bringing the phenotype into being, chromatin modifications, which are often referred to as epigenetic changes, affect the local kinetics of gene expression. They stabilize cellular states and are rarely transmitted to offspring.

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Enhancers: Encoding Regulation Across Time

Shayne Easterwood and Tae Hoon Kim

Abstract Fundamental question in biology is how our cells react and adapt in an enduring manner. This requires the control of dynamic gene expression and the ability to make appropriate regulatory changes based on ever evolving physiological contexts. As the primary regulators of the genome, enhancers orchestrate complex programs of gene expression across space and time. Enhancer activation is driven by multiple layers of epigenetic controls, allowing for persistent regulatory changes that enable anticipation across cellular lineages, organismal development, and generations. Non-coding RNAs transcribed from enhancers may serve as the functional unit of enhancer-driven anticipation, acting as a type of cellular memory to modulate the regulatory potential of the genome.

Keywords Enhancer RNA · eRNA · Non-coding RNA · Cis-regulatory elements · Transcription regulation

1 Introduction

The central dogma of biology—DNA is transcribed into RNA which is translated into proteins—describes essential steps of gene regulation in all life. Our past efforts have been toward achieving a complete mechanical and physical description of these systems based on steady state and equilibrium-driven models devoid of memory or persistence of information beyond genes. Anticipation at the biological level is how our cells "learn" and adapt to better prepare for possible future scenarios. In this context, anticipation can be appreciated in epigenetic programs of the genome, a process cultivated and refined through billions of years of evolution; a beautiful and intricate symphony coaxed from a jumble of DNA.

Each of the 37 trillion cells in a human body contains the same basic genetic information. Yet, each of those cells utilizes that information in a unique and specific way, producing the astonishing phenotypic diversity seen across the hundreds of different

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cell types. Enhancers are the key to this diversity. Enhancers are a class of genomic regulatory regions that amplify target gene expression in a precise temporal and quantitative manner, acting as the master regulators of complex programs of gene expression within the cell. Enhancers are responsible for coordinating the gene expression programs involved in development and differentiation, as well as those in response to stimuli. Much of enhancer activity is regulated by epigenetic controls, including histone modifications, DNA methylation, and post-transcriptional RNA modifications, enabling dynamic gene control. A critical component of enhancer function is non-coding RNA derived from enhancers. These enhancer RNAs (eRNAs) may potentially serve as a type of cellular memory to modulate the regulatory potential of the genome, thereby acting as the functional unit for enhancer-driven anticipation.

2 Enhancers

Genes get all the glory when it comes to discussions of DNA, but genes only account for 1–2% of the human genome [1]. Enhancers dramatically outnumber genes in the genome, both in terms of genomic real estate and quantity. More than two million candidate enhancers have been identified in the human genome, relative to just 20,000 protein-coding genes, and account for about 7% of the genome in total [2, 3]. Enhancers enable the real magic of the genome, acting as the principal regulatory agents, directing complex and dynamic gene expression programs that produce the infinite complexity of life [4–6]. Enhancers are *cis*-regulatory elements (CREs), distal to their target genes, often residing thousands or even millions of bases away in the genome, acting via concerted physical interaction with their target promoters. Multiple enhancers can regulate a single gene, and multiple genes may be regulated by a single enhancer, creating an interconnected multiplicity that enables enhancers to act as the conductors of their elaborate symphony of gene regulation, directing precise spatiotemporal control [7–9].

Active enhancers in the genome are identified by a signature of histone modifications (H3K4 mono-methylation and H3K27 acetylation), chromatin accessibility (nucleosome-depleted, nuclease-sensitive), dense clusters of transcription factor binding sites (TFBSs), and transcriptional activity [10, 11]. The combination and spatial organization of the TFBSs are what confers enhancer specificity [12, 13]. Three-dimensional genome architecture helps govern interactions between enhancers and promoters, as in most cases, enhancers and their target genes reside within the same topologically associating domain (TAD), defined by insulating CTCF boundaries [14, 15]. See Fig. 1. The very definition of an active enhancer is dynamic and driven largely by epigenetics, allowing for the genetic plasticity required for anticipatory processes. Interestingly, even promoters and silencers, which normally function distinctly from enhancers, can act as enhancers in different cell types or contexts [15–17].

Enhancers are functional regardless of orientation or location, and can be located upstream, downstream, or within a target gene [18]. As enhancers direct differentiation transcriptional programs, enhancer activation is highly cell type-specific, driven

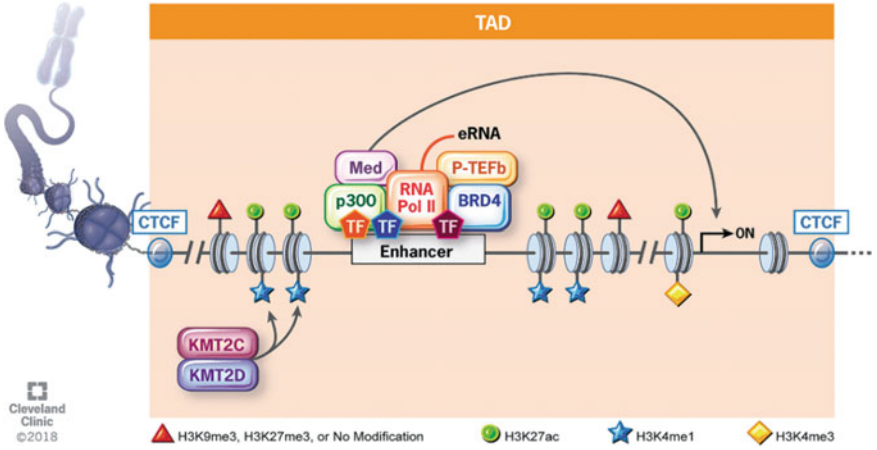

Fig. 1 General topography of an active enhancer [18]

by lineage-specific transcription factors (TFs) [19]. Clusters of proximal, adjacent enhancers can act in concert as a super-enhancer to regulate a common set of genes [20]. In instances of gene regulation by multiple enhancers, there is usually a "primary" enhancer, often the most distal of the enhancers relative to the target promoter, that functions as an anchor to establish contact with the promoter and acts to propagate the regulatory signals of the remaining enhancers [21–23].

While the genomic definition of an enhancer is primarily comprised of dynamic components, some enhancers remain constitutive while others are activated (induced) by a stimulus [24]. A genome-wide study on enhancer activity found that enhancers within a cell could be classified in three ways based on independent enhancer activity and local chromatin configuration: *classical enhancers* contain all classical chromatin indicators of an active enhancer and exhibit strong enhancer activity regardless of position or orientation, *chromatin-dependent enhancers* contain classical chromatin indicators of an active enhancer, but exhibit low independent enhancer activity, and *closed-chromatin enhancers* contain few classical chromatin indicators of an active enhancer, but exhibit independent enhancer activity [25]. Accordingly, super-enhancers typically consist of one classical enhancer with an array of additional chromatin-dependent enhancers, with the classical enhancer functioning as the anchor to coordinate the group of enhancers.

Enhancers as regulators of gene expression are ubiquitous throughout metazoan biology and beyond, as there is evidence of regulatory regions in bacteria that modulate gene expression by DNA looping [26–28]. Even plants have evolved transcriptional enhancers [29]. As enhancer regulation is essential for proper coordinated gene expression, high conservation of enhancers across species would seem logical. However, sequence conservation of enhancers is surprisingly low, with most enhancer sequences being species-specific. Further, the high mutation rates among enhancers may be a primary driver of species evolution, with recently evolved enhancers being

preferentially associated with genes under positive selection [30]. Despite sequence divergence, there appears to be a deep level of functional enhancer conservation across metazoan evolution, particularly those involved in development and differentiation. This has been demonstrated by transgene reporter assays using enhancers containing divergent sequences from distantly related species driving similar gene expression patterns across species [31]. Therefore, while transcription factor binding may account for the specificity and activity of enhancers, it may do so in a more nonspecific way [25, 32].

3 Enhancers and Transcriptional Regulation

The exact mechanisms by which enhancers function have yet to be fully elucidated, most often being described in broad generalizations. The prevailing and well-accepted model is that enhancers, which are typically linearly distant from their target genes in the genome, come into close proximity to their target gene promoter. This is accomplished by chromatin looping facilitated by Cohesin and anchored by a large protein complex composed of a unique constellation of transcription factors, coregulators, chromatin modifiers and readers, and components of the transcriptional machinery (including RNA polymerase II and other transcriptional activators). See Fig. 2.

While enhancer-promoter looping is considered to be a requirement for enhancer activity, the details involving the precise sequence of protein assembly that leads to looping (which can include hundreds of proteins) and the actual transcriptional trigger are still unclear, though we know the major players [28]. As mentioned, transcription factors are the key to enhancer specificity, though, how that specificity is enacted is less apparent. Transcription factors can bind and congregate at enhancers in different ways and for different purposes, in terms of their contribution to enhancer-promoter looping and overall enhancer function. Lineage-specific transcription factors may

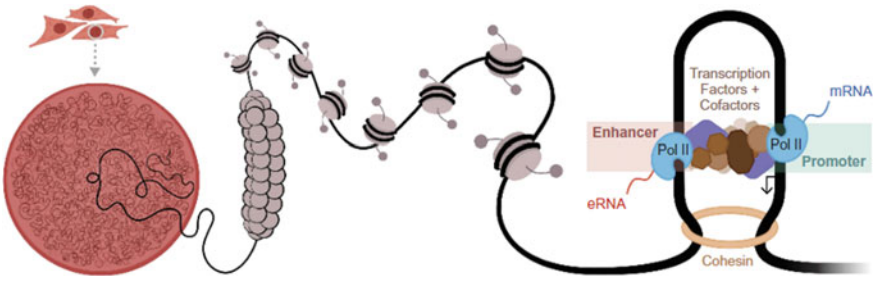

Fig. 2 General model of enhancer-promoter looping [35]

act as a "pioneer" by binding nucleosome-occluded enhancers, displacing the nucleosome, thereby making the enhancer accessible for subsequent transcription factor binding and enhancer activation [33].

There are three main models of transcription factor binding at enhancers: "TF collective," "billboard," and "enhanceosome", and enhancers may exhibit one or a combination of these binding models. See Fig. 3. In the *TF collective* model, transcription factors may bind the enhancer directly or indirectly (meaning they may bind the DNA directly or may bind to another transcription factor that is bound to the DNA). There is less evolutionary sequence conservation in this model, with more flexible number, type, and order of binding motifs. In the *billboard* model, all transcription factors bind the enhancer directly, and evolutionary conservation of each motif is high, though the order of those motifs within the enhancer is more flexible. In these first two models, cooperativity between transcription factors is indirect, meaning the transcription factors do not interact with each other, rather they work together to achieve a common goal (such as nucleosome displacement or specific coactivator recruitment). In the *enhanceosome* model, the transcription factors all bind the enhancer directly and cooperate directly with each other. In this model, the motif types and order are more highly conserved and structured. Adding an additional layer of complexity, the motif "grammar" among enhancers can vary, with parameters involving the type of motif; the binding affinity of the particular motif for the cognate transcription factor; the number, order, spacing, and orientation of the motifs; as well as the local DNA secondary structure at the location of the motif.

Transcription factors bound to the enhancer then go on to recruit coactivators, chromatin readers, modifiers, and remodelers, and enzymatic components of the transcriptional machinery, including RNA polymerase II (RNAPII); all integral to enhancer-promoter looping and/or transcriptional activation. While the transcription factors bound to enhancers may be lineage or stimulus-specific, much of the rest of the proteome involved in enhancer-promoter interactions tend to be more generalized

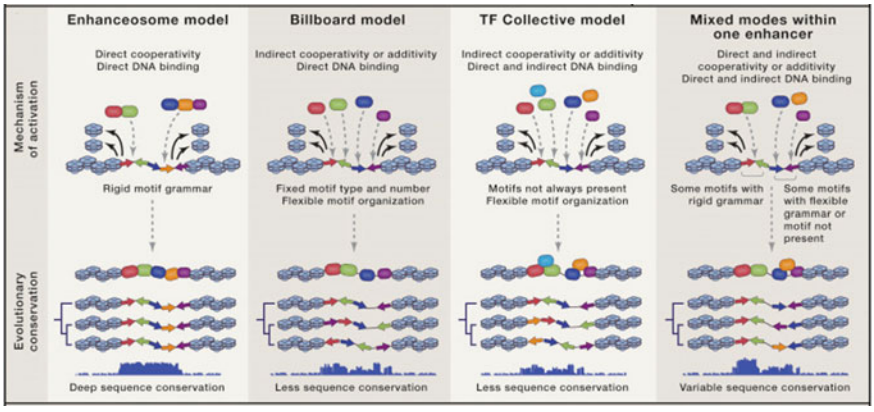

Fig. 3 Different models of TF binding and cooperativity in enhancers [37]

across active enhancers in the genome. Or, at least common across enhancers that regulate a core group of genes, such as those activated by steroid hormone receptors, like Estrogen Receptor alpha [34, 35]. Some of the more well-known components of the enhancer-promoter proteome include histone modifiers CBP, p300, and MLL3/4; architectural proteins Cohesin and CTCF; coactivators Mediator, Integrator, p-TEFB, and BRD4 that contribute to the regulation of transcription; and, of course, RNAPII and associated cofactors. A large number of musicians contribute to our enhancer-conducted symphony, and perturbations of any of them will disrupt the harmony. Indeed, the histone acetyltransferase p300, essential for enhancer function, is one of the most commonly mutated genes across cancer types [36, 37].

Enhancer activation and repression are enacted by an interplay between the binding of coactivators and repressors, deposition of activating or repressive histone modifications, and dynamic DNA methylation [38–40]. Interestingly, DNA methylation is strongly implicated in epigenetic inheritance (or "memory") and is a primary means of anticipation at the genomic level [41]. It's not surprising it plays a role in the modulation of the genome's primary regulator of gene expression.

Transcription across the genome has been demonstrated to occur in "bursts," with transcriptional burst size (duration) and frequency determining the overall amplitude of gene expression [42]. Enhancers have been implicated in regulating burst frequency, but not size, which calls into question a common aspect attributed to the mechanism of enhancer function [43]. The requirement of a rigid physical enhancer-promoter contact for transcriptional activation has been rather dogmatic in the field, with a variety of early studies supporting this hypothesis [28]. However, recent studies are challenging the notion of rigid enhancer-promoter contacts and have demonstrated that enhancers and their cognate promoters are not always in direct physical contact during transcription [44]. Based on these observations, a new model has been developed whereby enhancer-promoter contacts are dynamic and transient, by requirement, and successive contacts would lead to increased frequency of transcriptional bursts [28]

4 Enhancer RNAs

Target gene transcription is not the only transcription occurring at enhancer-promoter contacts. The majority of enhancers are also transcribed into non-coding enhancer-derived RNAs (eRNAs). Generally, eRNAs originate from a transcription start site (TSS) near the center of the enhancer (where the clusters of TFBSs are typically located) and may be synthesized from either strand [45]. Initially, bulk RNA studies led researchers to believe eRNAs are transcribed bidirectionally. However, a recent seminal single-cell study on nascent RNA transcription has shown that transcription is generally exclusively strand-specific for each individual cell [45]. That same study also showed that while bulk studies report that eRNA transcription is low relative to target mRNA transcription, eRNA transcripts are expressed at levels similar to target mRNAs, but in a more restricted subset of cells. The temporal dynamics of

eRNA transcription relative to mRNA transcription have not been clearly resolved, as studies have shown conflicting results, indicating that eRNA transcription either precedes or occurs simultaneously with target mRNA transcription [46–49]. The reality is likely somewhere in the middle, where either or both scenarios are true, depending on the context and enhancer-promoter pair studied.

eRNA transcripts display broad heterogeneity in sequence, length (ranging from 200 bp to 4 kb), and polyadenylation, but are typically capped, unspliced, and restricted to the nucleus [10, 50–54]. Most eRNAs are relatively short-lived, with stability mediated by susceptibility to degradation by nuclear RNA exosome [53, 55]. eRNAs are also subject to post-transcriptional modifications, specifically 5-methylcytosine (m^5C) and N^6-methyladenosine (m^6A), both of which are involved in the recruitment of transcriptional coactivators and may also modulate the stability of the eRNA [56, 57]. While there is no consensus secondary structure common to all eRNAs, there are subsets of eRNAs with secondary structures similar to other categories of functional non-coding RNAs such as miRNAs, snRNAs, tRNAs, and lncRNAs, indicating probable functional relevance [54]. In addition to the cell-specificity of active enhancers themselves, eRNA transcription from a common enhancer also displays differential activity across cell types and contexts [58].

The function of eRNAs is currently the subject of intense debate. Originally thought to be a simple byproduct of RNAPII activity at the promoter of the target gene, eRNAs have been repeatedly demonstrated to be critical for proper enhancer activity. Transcriptionally active enhancers tend to be more potent, with the level of transcription proportional to the potency of the enhancer [59]. Inhibition of eRNAs (both pre- and post-transcription) consistently results in a reduction in transcription of the target gene(s) [54]. Interestingly, when a target gene is regulated by multiple eRNAs, inhibition of one of the eRNAs often results in increased transcription of other eRNA(s) in the regulatory network, suggesting a compensatory mechanism and coordinated action of eRNAs [9, 60]. This redundancy speaks to the evolutionary importance of eRNAs to the proper functioning of genomic expression.

While the exact mechanism of action of eRNAs has not been determined, there are several non-mutually exclusive hypotheses. The prevailing ideas include: stabilizing transcription factor binding at the enhancer (RNA trap); facilitating the formation of the protein complexes that promote enhancer-promoter looping through direct interactions with essential enhancer-promoter contact proteins such as Mediator and Cohesin; mediating chromatin accessibility through direct interactions with histone modifiers; and regulation of the transcriptional machinery [54, 61–65]. While much of the research has focused on eRNAs functioning in *cis,* while still tethered to the local chromatin, several studies have also demonstrated that some longer, more stable, polyadenylated eRNAs transcribed from super-enhancers act in a regulatory capacity in *trans* (at alternate chromosomal locations) [4, 66, 67].

It may not be that enhancers and enhancer RNAs escape precise mechanistic definition so much as an issue with enhancers being studied in bulk. There are likely many different types and categories of enhancers, that while having similar characteristics, may act in different ways, and a single enhancer may function via multiple mechanisms, depending on the state or context of the cell.

5 Enhancers and Phase Dynamics

Searching present-day technical models for an analogy of how the human brain receives signals from the heart leads to a base model for interaction between open systems: the Open System Interconnection Basic Reference Model (OSI), which assumes a seven-level organization of data transfer [42]. Here, each level serves its own part in the interconnection process. The "levels" organization is an important characteristic of the model. When a Phase dynamics may play an important role in how an enhancer functions. Liquid–liquid phase separation (LLPS) resulting in the formation of condensates inside the cell is widely reported to play an integral role in many cellular processes, including transcription [68]. LLPS and resultant condensate formation is a process driven by multivalent electrostatic interactions between participating macromolecules (primarily nucleic acids and proteins) that result in high local concentration, initiating oligomerization leading to coalescence into a high-density liquid droplet that is effectively sequestered from its surroundings [68, 69]. Biomolecular processes that form LLPS condensates exhibit high efficiency and fidelity via organizational and architectural specificity attained by condensate formation [28, 70]. Condensates can dynamically assemble and dissolve based on the relative concentrations of participating macromolecules. Further, while condensates often contain up to hundreds of participating macromolecules, there is often just a small subset of these components that are essential for condensate formation [70]. An important characteristic of these condensates is the control of molecules into and out of the condensate, which contributes to the precise regulation of the cellular process [71]. Due to the concentration of selective macromolecules and the specialized processes that are facilitated by condensate formation, as well as their distinct separation from the surrounding environment, these condensates are often also referred to as membraneless organelles [72]. Cellular condensates are not a new concept and include well-known and studied examples throughout the nucleus and cytoplasm, such as nucleoli, P-bodies, nuclear speckles, and stress granules, but the idea that dynamic condensates may control a majority of cellular processes is fairly recent and gaining significant traction [70, 73].

During transcription, the components of the enhancer-promoter loop (including Mediator, transcription factors, coactivators, and RNAPII along with local RNAs and multivalent DNA at enhancers) form a condensate [74–77]. See Fig. 4. Transcription regulation mediated via condensate formation provides a logical framework for overall enhancer function, with condensate formation establishing the scaffold which enables many of the complex behaviors exhibited by enhancers. For example, transcriptional condensates can facilitate the complex architectural task of aggregating multiple enhancers and genes into a common regulatory network. See Fig. 5. Additionally, a proposed model of enhancer-promoter "kissing" across the surface of a transcriptional condensate provides the mechanism behind dynamic and transient enhancer-promoter contacts and transcriptional bursting [78].

Based on the overwhelming evidence that RNAs play an integral role in LLPS, providing ample opportunity for multivalent interactions, it is likely that eRNAs

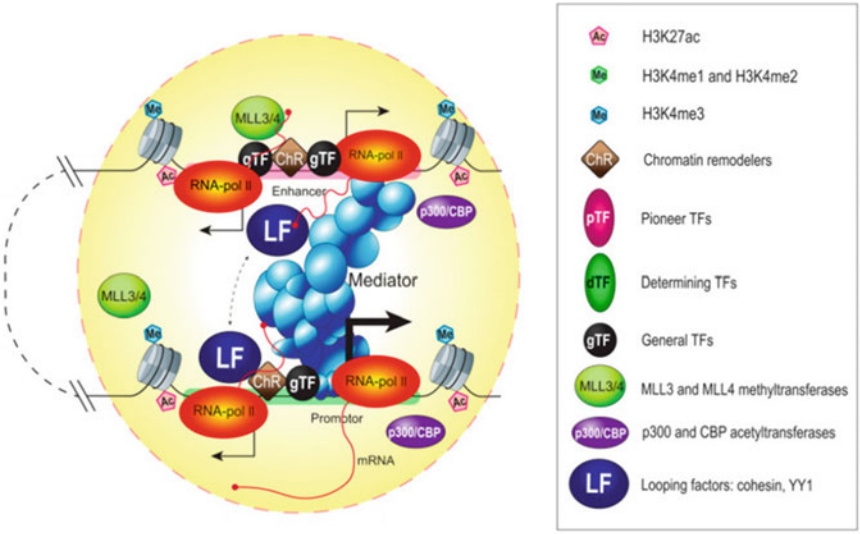

Fig. 4 Model of a transcriptional condensate [81]

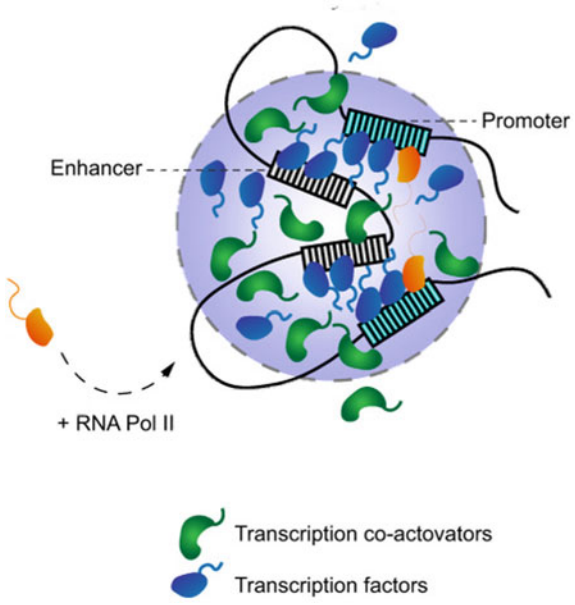

Fig. 5 Model for sequestration of multiple enhancers within a transcriptional condensate [76]

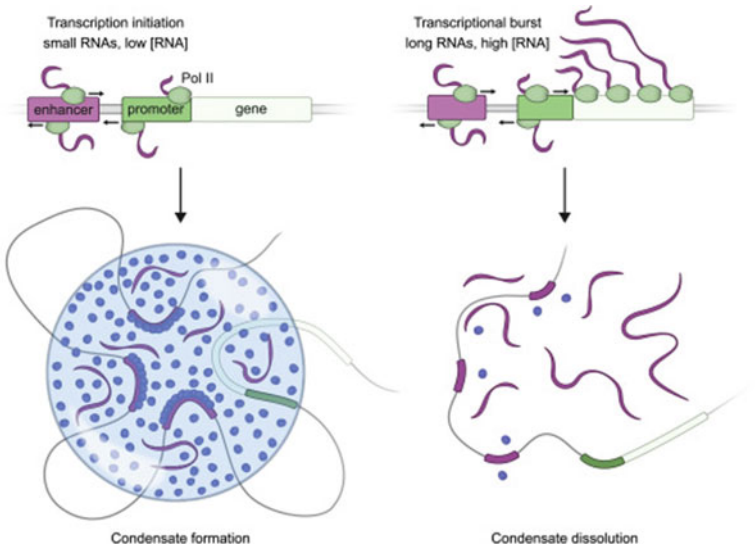

Fig. 6 Model of dynamic condensate formation mediated by RNA concentration [86]

are important and specific contributors to transcriptional condensate formation [79, 80]. Specifically, m^6A post-transcriptionally modified eRNAs have been demonstrated to modulate condensate formation [81]. Further, a model of transcriptional regulation has been suggested whereby local RNA concentration (including both eRNA and mRNA transcripts) modulates condensate formation by a self-feedback loop, triggering condensate dissolution when a threshold of RNA concentration has been reached [82]. See Fig. 6. In addition to their potential role in mediating the formation and dissolution of condensates, eRNAs could also influence the composition, stability, and behavior of the transcriptional condensate through their unique electrostatic contribution. Conversely, while eRNAs may contribute to condensate formation and function, the condensates themselves may also serve to mediate the stability of eRNAs by sequestering them from nuclear RNA exosome degradation [83].

6 Enhancers and Anticipation

Enhancer activation is primarily orchestrated by epigenetics in an inherently dynamic process that enables the anticipatory processes in the cell. The duration of selective enhancer activation is impacted by cellular context and shapes the genomic regulatory environment in an enduring manner. eRNAs are the logical functional unit of enhancer-driven anticipation, as they are intimately involved in nearly every aspect

of enhancer-driven transcriptional control, from establishing enhancer-promoter contacts to modulating transcriptional condensates.

Unpublished data from our lab suggests that eRNAs are subject to dynamic stability based on cell context, where we observed that an oncogene-associated eRNA displayed increased stability in cancer cells, relative to the same eRNA transcribed in non-cancerous cells. Dynamic eRNA stability and persistence within a cell regulate the duration with which enhancers can exert their genomic regulatory control. Therefore, processes that modulate eRNA stability underpin the role enhancers play in anticipation. Sequestration in a condensate and post-transcriptional modifications (such as methylation and polyadenylation) are conditions that can impact the stability and persistence of eRNAs and are dynamically modulated by cell context. Biomolecular condensates can even progress from liquid-like characteristics to a more gel-like consistency, conveying enhanced stability within the cell, and extending the duration of the processes coordinated by the condensate, including transcription and enduring eRNA stability [84]. It is even possible that sequestered eRNAs can be secreted and passed between cells via extracellular vesicles, able to exert their regulatory influence in neighboring cells [85]. Further, small RNAs have been implicated in epigenetic inheritance, so it is also possible that eRNAs play a role in RNA-mediated transgenerational inheritance, potentially extending their regulatory influence to future progeny [86].

7 Enhancers and Human Disease

By aligning with anticipation, science and medicine can better focus on mitigating disease and promoting health and wellness in a way that works *with* the body, instead of playing a dangerous, and often futile game of whack-a-mole with diseases and pathogens. Many current treatments and therapies come with an unsavory host of pleiotropic effects, that are often detrimental in their own right, as they fail to account for how different cells utilize gene products in different and often opposing ways, opting instead for a cell-type agnostic shotgun approach. Utilizing cell-type and condition-specific enhancers and eRNAs provides a wide-open platform for harnessing a hugely powerful component of the body's anticipatory system, enabling the development of finely-tuned targeted therapies while limiting harmful side-effects.

Genome-wide association studies have shown that genetic variation in enhancers accounts for 60–80% of disease-associated single nucleotide polymorphisms, far outnumbering risk variants mapping to coding regions of the genome [87, 88]. Since CRISPR and other genome-editing technologies have not yet matured to the point of human utility, genomic sequence manipulation is beyond our control. As such, the focus should be on targeting enhancer epigenetics and cell type-specific eRNAs. The cell-specificity of eRNAs has been estimated at greater than 30% overall, and closer to 50% for super-enhancers [89, 90]. However, these estimates are likely low, as the majority of studies are conducted on cells in a steady state and do not account for

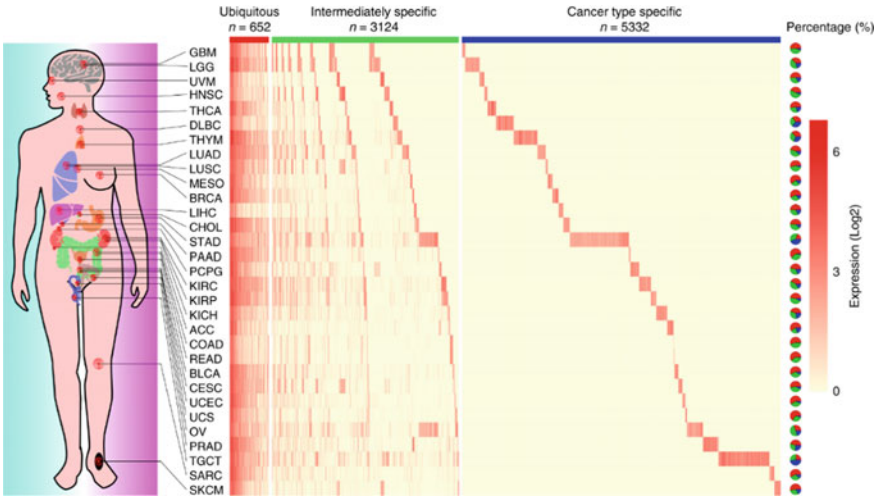

Fig. 7 Analysis of enhancer RNA transcription across human cancer types [91]

eRNAs induced by a condition or stimulus. Accordingly, cancer researchers have long suspected that eRNAs hold an elusive promise for cancer therapies, and computational studies abound that tout the potential for targeted treatments via cancer-specific eRNA transcription [91]. See Fig. 7.

However, outside of cancer research, cell type-specific eRNAs seem to be curiously overlooked, and very little headway has been made in developing therapies targeting enhancers [88]. Analysis of the data from the FANTOM5 project, which sought to identify enhancers in different human tissues and cell types, determined that immune, neuronal, neural stem cells, and hepatocyte cell types exhibit the highest ratios of cell type-specific enhancers and show a high ratio of enhancers to genes, offering a rich buffet of cell type-specific eRNAs from which to sample [50]. This high specificity provides a myriad of opportunities to explore how enhancers can be utilized for therapeutic benefit.

There are nearly endless opportunities for potential eRNA therapy, where cell type-specific fine gene control is desirable. Targeting eRNAs to mitigate lung damage sustained during severe Influenza infection is a possible application. In severe Influenza infection, an overzealous immune response resulting in a "cytokine storm" can lead to acute respiratory distress syndrome (ARDS), which has a high mortality rate [92, 93]. The development of ARDS from severe Influenza infection has been correlated with abnormally high levels of TRAIL, an immune system secondary response cytokine [94]. TRAIL is expressed by many immune cell types and is an essential component of the innate immune response to viral infection, required for proper viral clearance by inducing apoptosis (cell death) in infected cells [95]. During an Influenza-induced cytokine storm, TRAIL is overexpressed in the lungs resulting in the apoptosis of neighboring uninfected epithelial cells which causes a disruption in the alveolar barrier and dysregulated fluid clearance from the lungs,

leading to ARDS. Interestingly, TRAIL is not overexpressed by *all* immune cells in this context; macrophages, specifically, are the singular perpetrators of excessive TRAIL expression [96, 97]. So, the question becomes, how can TRAIL expression be mitigated in a single cell type? The use of antibodies would affect TRAIL expression in all cells, potentially inhibiting viral clearance and extending the timeframe of infection (an undesirable side effect) [95, 98]. However, if macrophages recruited to the lungs during Influenza infection transcribe cell type-specific eRNAs that regulate TRAIL gene expression, then theoretically, those cell type-specific eRNAs could be targeted to selectively modulate TRAIL gene expression in just macrophages, leaving TRAIL expression in other immune cell types at endogenous levels, to do its job as intended.

Another hypothetical scenario considers an alternative way of targeting eRNAs in a more broad application. The innate immune system is classically considered as the nonspecific first responders to an infection, conveying no lasting "memory" or extended protection. This is in contrast to the cells of the adaptive immune system which produce antibodies against a specific antigen, conveying enduring protection. Surprisingly, the innate immune system does indeed convey a type of nonspecific memory, conferring enhanced protection against re-infection or subsequent infection after an initial infection in some instances; perhaps a rather underappreciated anticipatory process, overshadowed by the specificity of the adaptive immune system. It turns out that this innate immune system memory is facilitated by epigenetic modifications associated with enhancers (H3K4 methylation and H3K27 acetylation) [99]. What if we could induce or extend that protection by activating or prolonging associated enhancer activity or by supplementing with the correlated eRNAs?

Yet another scenario could involve utilizing the different chromatin states, determined by epigenetic modifications, of different subsets of enhancers for therapeutic benefit. As detailed earlier, local chromatin configuration at enhancers plays a key role in their activation. Research has demonstrated that in an inflammatory response, rapid early response genes have constitutively permissive chromatin at their enhancer regions, whereas secondary response genes require chromatin remodeling at their enhancers before gene expression is activated [100]. Researchers have exploited this chromatin differential to selectively inhibit eRNA transcription at induced de novo active enhancers that regulate secondary response genes through inhibition of the BET family of proteins, which includes BRD4, a common component of the enhancer-promoter proteome that assists with the recruitment of RNAPII. This has been demonstrated in a variety of disease (and simulated disease) states [101]. While BRD4 has been implicated as a common component of the enhancer-promoter proteome, these studies demonstrate that BRD4 inhibition is able to selectively affect transcription at secondary response genes, while having no effect on early primary response genes. This could provide a way to modulate coordinated groups of enhancers specific to the cell context.

Finally, if eRNAs can regulate transcriptional condensate formation, either through local RNA concentration or via post-transcriptional modifications, target gene expression could be selectively modulated via eRNA-directed condensate control. Additionally, if the condensate itself acts to regulate eRNA stability and

persistence in the cell, then eRNA-directed condensate control has the potential to function as a self-feedback loop, either limiting or promoting enhancer activity via dynamic control of eRNA stability.

8 Final Thoughts

Enhancers are at the very core of biological anticipation; the master regulators of gene expression, conducting a seemingly impossibly complex and intricate symphony, unique to each cell. Every aspect of enhancer function is dynamically controlled by epigenetics, from enhancer activation to the formation of transcriptional condensates. The multiple and interconnected layers of epigenetic controls enable the flexibility to enact anticipatory processes; to allow cells to adapt and exist in a state informed by the past. Enhancer RNAs may be the key to unlocking the power of enhancers and harnessing their role in anticipation in a highly selective manner.

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Mechanistic Basis of Regulation of Host Epigenetic Landscape and Its Association with Immune Function: A COVID19 Perspective

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Abstract In the twenty-first century emergence, the rapid spread and pathogenicity associated with SARS-CoV-2 have put a tremendous impact on the human population leading to the COVID19 pandemic. Several theories, seminal findings, and mechanistic evidence have been proposed to understand the pathogenesis, origin, host immune response, and therapeutic approach. Although a coordinated effort by several countries enabled the vaccination drive to be successful, there is still a large gap between host epigenetic architecture and virus, which necessitates a deep understanding of the molecular basis of epigenetic interplay between virus and host modulating immune function. It is noteworthy to consider that virus-induced alteration in chromatin marks, especially in histone and DNA, plays an essential role in driving immunopathogenesis. In this backdrop, several question marks arise: how the phenomenon occurs, what modifications are altered, how it is associated with immune function, and what epigenetic modulators could be adapted in clinical settings are poorly understood. In this chapter, we have discussed the cutting-edge aspect of the epigenetic basis of immune function and its current advancement for better therapeutic options in a clinical setting.

Keywords Inflammatory signaling · Epigenetic modulates · Innate immunity · Adaptive immune response

1 Introduction

The emergence and rapid global spread of novel highly pathogenic SARS-CoV2 have created a severe health crisis affecting a million lives with an adverse impact on several aspects of livelihood. The highly contagious SARS-CoV-2 infection and transmission become an ongoing challenge because of the evolving and re-emerging infectious pathogens. The modulation of the host cell's epigenetic landscape following the virus infection enlightens a molecular tool used by the viruses

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to antagonize cellular signaling. In the journey of the long-armed race, both virus and host display a complex mechanistic underpinning for successful infection and survival. Pieces of evidence show that epigenetic events like DNA methyation and post translational modification of histone tails play a prominent role in fine-tuning the transcriptional programme associated with anti-viral response and host immune evasion [1–4]. Understanding the inherent epigenetic mechanism governing the behavior and memory of innate and adaptive responses will unfold several unanswered questions. Therefore, in the current chapter, we provide a comprehensive description of novel epigenetic modifications determining the complex interplay between SARS-CoV2 and the host.

2 SARS-CoV2-2 Mediated Pathogenesis and Its Association with Host Immunity

Though the immune-Pathogenic phase of COVID-19 is still not wholly understood, previous and ongoing findings have elucidated that SARS-CoV2 pathogenesis is mediated by three phases: viral replication, immune hyperactivity, and pulmonary destruction [5]. The pathogenesis is exceedingly complex, yet undefined immune-mediated pathogenesis has exceptional heterogeneity and classical reciprocity at clinical, immunological, and viral levels [6]. Patients infected with SARS-CoV2 exhibit symptoms like pneumonia and severe symptoms of acute respiratory distress syndrome (ARDS) associated with multiple organ damage and failure. However, the incidence of several co-morbidities further complicates the recovery phase contributing to immune suppression [7]. As it is well known, SARS-CoV2 target cells through the S-protein that binds to ACE Receptor, replicating and assembling in target cells before extracellular release [8]. Inflammatory signaling molecules are released by infected cells and may induce multiple organ injuries through Innate and Acquired Immunity [9].

Entry of SARS-CoV2 in the human body is mediated by different organs leading to the alveoli in the lungs. Among the two types of cells lining the alveolar epithelia: Type-I cells and Type-II cells, the Type-II pneumocytes express the angiotensin-converting enzyme-2 (ACE-2) receptors. These receptors play a significant role in viral entry through specific binding with viral spike proteins. TMPRSS2 accompanies the initial binding mediated activation and cleavage of viral S protein, followed by the viral genomic RNA injection into the cytosol and after the viral replication. This process can hijack cellular machinery and induce viral genomic RNA synthesis, virion assembly, and mature virions released by exocytosis [10].

SARS-CoV2 entry inside the host cell-associated with inflammatory signaling by type-II cells, which recruit macrophages to release cytokines. This could lead to vasodilation and then permit more immune cells, principally neutrophils, to enter the infection site from the capillaries. These phases cause the fluid accumulation and dilution of the surfactant inside the alveolus, causing their collapse and thus

decrease in gaseous exchange [6]. Neutrophils' action and their release of reactive oxygen species (ROS) kill the infected cells. In further inflammation cases, the protein-rich fluid enters the bloodstream and causes systemic inflammatory response syndrome (SIRS), which leads to Septic Shock and thus multi-organ failure [11]. The majority of patients with COVID-19, more than ~ 80% of cases are asymptomatic or suffering from mild symptoms. Among them, ~ 30% can develop neutralizing antibodies against the viruses, and thus they can stay well. Another ~ 50% of patients develop mild to moderate symptoms lasting for 6–7 days more or less, and thus they eventually become immune and recover properly [6].

COVID patients have lymphopenia with an almost ~ 20% drop in lymphocyte count in severe cases. They show a marked reduction in $CD4^+$ T cells, $CD8^+$ T cells, and NK cell numbers compared to the mild cases. In contrast, patients with severe phenotype exhibit elevated cytokines, particularly IL-6, IL-1B, and IL-10, leading to a "Cytokine Storm" and inflammation-induced multiple organ dysfunctions [7]. Patients with severe disease are characterized by B-cell activation and exaggerated IgG response. Furthermore, patients with COVID-19 are characterized by innate immune hyper-activation, which is responsible for driving an acute lung injury, and adaptive Immune dysregulation, which leads to an increased risk of viral reactivation [12].

3 Epigenetic Modulates Host Innate Response

Impairments in associated epigenetic events like DNA methylation and histone modifications play a crucial role in regulating critical immune function associated with dendritic cell (DC), T cell, macrophage, and macrophage function [13]. Several activated immune cells such as T cells, macrophages, B cells, DC, NK. cells, neutrophils, and monocytes play a significant role in innate immunity and adaptive immunity [12, 14, 15]. Although these cells mediate their action by secretion of several soluble factors, overproduction and hyperactivation of the immune system could lead to a cytokine storm. Furthermore, the immune evasion adaption strategy adapted by SARS-CoV2 and other RNA viruses bypasses the immune network and modulates the pathogenesis [3, 5].

In response to SARS-CoV2 infection, host innate immune cells express different types of pathogen-related receptors mediated signaling leads to the production of chemokines or cytokines inducing cell death in infected cells [6]. Previous in silico and experimental evidence suggests SARS-CoV2 proteins induce TLR/RLR signaling, leading to the induction of pro-inflammatory immune response [7]. Studies have highlighted that RLR signaling shows to be indispensable for IFN production [1, 16]. In most of the TLR responsive inflammatory genes, open and permissive chromatin marks like H3K4me3 and histone H3 acetylation (H3Ac) are prevalent, as observed in the promoters of primary response genes (PRG) in both inducible and basal conditions [17]. Studies have also shown that adding histone marks like H4K5, H4K8, and H4K12Ac at the PRG promoter can recruit the BRD4 protein.

The binding of BRD4 allows the recruitment of positive transcription elongation factor, P-TEFb complex, to phosphorylate S2 at CTD of RNA Pol II, leading to active transcription of genes [18]. Previously it was also observed that H3K4me3 is associated with robust transcription of several genes regulating innate immunity in different cell types [19]. H3K4me3 marks are shown to correlate with strong GC content/CpG elements in the promoters [20]. In addition, SARS-CoV-2 proteins are shown to induce host immune response by activating the TLR signaling pathway. In several cell models like DC, PBMC, and macrophage, it has been observed that SARS-CoV2 proteins are recognized by several TLR members and pro-inflammatory trigger responses [21]. A robust and protective innate immune response requires the production of different IFN subclasses by T cells, pDC, NK cells, and other cellular populations, which in turn are further associated with the stimulation of interferon-stimulated genes (ISG), thus preventing the viral replication and limits viral spread and load [22]. Among interferon types-I, interferon was found to play a critical role in innate driven anti-viral defense. Among many transcription factors STAT1 family can upregulate the type I IFN genes. H3K4me3 was also involved in KMT2B induced PIGP mediated membrane anchoring of CD14 for TLR-4 mediated signaling [23].

Evidence has shown that type I IFN can induce lysine methyltransferase SETDB2, which upregulates the trimethylation of H3K9 at selected promoters of several genes associated with anti-viral defense [24]. In addition, IFN-γ, a type II interferon, is associated with the upregulation of H3K27Ac in the promoters of several genes like NF-α, IL-6, and IL-12 promoters [25].

Studies have demonstrated that both miRNA and lncRNA are differentially regulated and regulate the innate immunity genes in several cell types, including DC, followed by LPS stimulation [26]. In addition to this role, miRNA and lncRNA in the development of innate memory were explained by several studies [27]. mir221 and mir222 modulated the functional reprogramming of mouse bone marrow macrophages on LPS-induced tolerance [28]. miRNA-like Similar to this, the role of lncRNA and its implication in gene expression regulation in innate immunity have been explored by several studies [29]. LncRNA can facilitate the H3K4me3 epigenetic priming [30]. It is imperative to note that miR-146a, miR-155, and miR-9 play a key role in inducing TLR signaling, suggesting their prominent function in the acute phase of inflammation [31]. The role of NK cells is shown to be highly instrumental in mediating the innate and adaptive immunity as well as conferring innate memory during viral infection [32]. NK cells can release cytolytic granules and inflammatory cytokines, including IFN-γ and TNF-α. Long-lived memory-like characteristics in NK cells, including long-term persistence and enhanced responsiveness to pathogen infection and response to external stimuli, are the results of epigenetic alterations in the infected cells [33].

Furthermore, RNA-containing viruses can stimulate TLR3 signaling, leading to activation of NF-kβ and IRF3 genes, resulting in the endogenous pluripotency network and inducing epigenetic plasticity. Inconsistent with this evidence, results have shown on activation of NF-kβ interacts with HAT proteins p300 and CBP HP1 in the promoter region of several genes, characterized by increased H3K9me3 at the Sox2 and Oct4 and reversal of H3K9me3 at the Sox2 and Oct4 promoters thus

contributing an open chromatin state. These changes are associated with decreased expression of different HDAC family genes, H3K79 HMT Dot1L and H3K4 HMT Ash11 [34, 35].

Studies have also demonstrated that this epigenetic priming is associated with alterations in chromatin topology and regulates the expression of inflammatory cytokines and chemokines, which plays a significant role in conferring innate immune memory [34–36].

4 Epigenetic Basis of SARS-CoV2 Induced Adaptive Immune Response

The development of an adaptive immune response against SARS-CoV2 has been well investigated in several studies [37, 38]. The role of $CD4^+$ specific T cell responses was more prominent concerning the $CD8^+$ T cell in COVID19 cases. Seminal findings have shown in acute COVID19 cases that rapid induction of $CD4^+$ T cells is associated with mid phenotype and accelerated viral clearance. In contrast, the strikingly extended absence of $CD4^+$ T cells is associated with severe or fatal COVID19 [37–39]. Virus-specific $CD4^+$ T cells differentiate into Th1 cells, and T follicular helper cells (Tfh) play a significant role in anti-viral immunity by coordinating B cells and other immune cells. $CD4^+$ T cells in COVID19 patients mediate effectors' function by producing several cytokines like IFNγ, TNF, and IL-2 and are associated with protective immunity. In addition, SARS-CoV2 specific $CD8^+$ T cells in COVID19 patients exhibit efficient cytotoxic function by secreting granzyme B, perforin, IFNγ, and CD107a [40, 41]. Studies have shown that in patients with COVID19, I(g)M immunoglobulin followed by IgG spike protein-specific antibodies and followed an immune response within 7–10 days of post-infection [42]. The spike and nucleocapsid-specific IgG titers are highly predominant in patients with COVID19, where these antigens are a target for > 90% of neutralizing antibodies. However, the finding has shown that injection of a high dose of neutralizing antibody in SARS-CoV2 infected cases has limited effects on COVID19 and indicates the requirement of effective T cell response to clear the infection [43]. Nevertheless, a coordinated response encompassing $CD4^+$Tcell, CD^+ T cell, and antibody response is highly prerequisite for efficient immune response.

T cells, NK cells, and B cells play a key role in mediating memory response against subsequent infection and clear cytopathic viruses. Memory B cells can respond to reinfections and induce the growth of plasma cells, which continue to secret antibody and causes serological memory. Studies have shown that memory B cells play a durable immune response compared to the antibody for long-term immunity against SARS-CoV2 infections. Previously it was observed that SARS-CoV2 induced humoral response is relatively short-lived, and memory B cells disappeared after the primary infection [44]. In contrast, $CD8^+$ memory T cells and, to some extent, $CD4^+$ memory T cells exhibit 6–11 years, suggesting a long-term immunity

[45]. Furthermore, in convalescent patients with mild symptoms, the SARS-CoV2 specific durable humoral and cell-mediated immune response was seen for up to 7 months [46].

The mechanistic basis governing B cell differentiation into plasma cells and secretion of antibodies is poorly understood. Permissive histone modifications like H3K4me1 and H3K4me4 associated with gene promoters and enhancers play an essential role in B cell development. Evidence has shown that upregulation of cl6, Pax5, and Spib genes play a crucial role in inhibiting plasma cell differentiation by deacetylation of these promoters [47, 48]. However, the expression of Blimp-1, a transcriptional repressor in one aspect, recruits HDAC and down-regulates the expression of Bcl6, Pax5, and Spib. In another aspect, Blimp-1 interacts with H3K9 methyltransferase G9a and likely recruits this to the *Pax5* and *Spib* promoters. Thus, Blimp-1 plays a crucial role in regulating plasma cell differentiation. Current evidence by Wauters et al. 2021, shows the role of Blimp-1 in plasma cell differentiation in a patient with COVID19 [49].

In addition, the differentiation of memory B cells is characterized by different histone marks [50]. It has been observed that EZH2 plays a significant role in memory B cell formation and antibody response [51]. EZH2 was found to catalyze H3K27me3 through the SET domain. In addition to EZH2, the role of istone acetyltransferase monocytic leukemia zinc finger protein (MOZ) can target the H3K9 and play a role in B cell memory formation. Current evidence by Yuannyuan et al., 2020, shows EZH2-mediated H3K27me3 methylation at the ACE2 promoter inhibits ACE2 expression.

Furthermore, the role of EZH2 in patients' COVID19 has been investigated by subsequent studies [2, 52]. In addition to this, both plasma and memory B cells are characterized by alterations in DNA methylation. Previous evidence shows a significant expression of DNMTs like DNMT3a in both plasma and memory B cells [53]. Although several lines of evidence have shown the implication of DNA methylation mediated epigenetic signature in patients with COVID19 [54, 55], B cell-specific DNA methylation, the role of DNMT in B cell differentiation in SARS-CoV2 infected patients is lacking.

A hypermethylated state characterizes Naïve $CD4^+$ T cells compared to the memory T cells. Findings have shown that hypomethylation of genes like *CCR6*, RAR-related orphan receptor C (*RORC*), the gene for ligands for P-selectin, E-selectin are hypomethylated are prevalent in $CD4^+$ memory T cells [56]. In contrast, in $CD8^+$ memory, T cells are characterised by low level of DNA methylation at the IFNG and IL2 promoter region. Similarly, memory T helper 1 (T_H1) and T_H2 cells exhibit hyperacetylation of histones at the promoters of IFNγ and IL-4 genes, whereas $CD8^+$ memory T cells are characterized by hyperacetylation of the promoter of genes of cytokines and effector molecules [57]. In addition to this, bivalent chromatin marks (H3K4me3 and H3K27me3 at the same region) associated with both open and close chromatin are associated with the $CD8^+$ memory cell. The previous study has also highlighted the potential role of EZH2 and PRC2 complex in regulating memory T cell differentiation [58]. The current finding shows that the role of EZH2 is highly crucial in virus-specific $CD4^+$ T cell expansion by inducing mTOR signaling [59].

In contrast to acetylation and methylation, although the role of other post translational modifications has been elucidated in regulating T cell memory, the detail mechanistic basis is poorly understood. A recent finding from single-cell chromatin accessibility and transcriptomic profile shows that alterations of the epigenetic landscape are associated with T cell inflammatory states and defective function of $CD4^+$ T cells in patients with COVID19 [60]. In support of this notion, several studies have elucidated the deregulated epigenetic profile and its association with T cell function [59, 60, 62]. Further studies are required to explore the epigenetic basis of T cell memory during SARS-CoV2 infection [21, 61–63].

5 Epigenetic Basis of Anti-Viral Immunity During SARS-CoV2 Infection

As far as the arms race between the virus and the host is concerned, alterations in the host immune-epigenetic architecture are prominent during an infection. In such a case, either the virus won by developing its strategy, or the host can defeat the virus life cycle in another case. Earlier findings have shown that innate immune responses are crucial in conferring anti-viral immune response, which is associated with alterations in chromatin remodeling or genome organization events. The IFNβ promoter is found to be associated with Sendai virus (SeV) infection [64]. Expression of IFNB can be induced by chromobox 2 (Cbx2), a polycomb chromobox protein, by recruitment of demethylase Jmjd3 to the *Ifnb* promoter facilitating H3K27me3 demethylation [65]. Histone modifications are associated with the expression of IFN, TNF, and ISGs in patients with COVID19. Transcription activating histone modifications like H3K4me3, H4Ac, and RNA Polymerase II occupancy decreases in ISG epigenetic landscapes compared to the IFN and TNF. In fact, the expression of ISG requires chromatin remodel complex like SWItch/sucrose non-fermentable (SWI/SNF) required for initiation of transcription [66, 67]. These histone marks and chromatin modifiers participated in the innate immune response against SARS-CoV2 [68]. The previous finding suggests hepatitis B virus (HBV) infection can be restricted through epigenetic mediated repression of the viral cccDNA [69]. In support of this notion, silent mating type information regulation 2 homolog 3 (SIRT3), a histone deacetylase, causes the deacetylation of H3K9 resulting in the augmentation of the H3K9me3 and decreasing H3K4me3 thus restricting viral replication [70]. In addition to histone acetylation, citrullination of histones at H3 (Cit-H3) was observed in patients with COVID19. Cit-H3 is associated with the neutrophil extracellular trap (NET), which is characterized by decondensed chromatin and positively correlated with increased cytokine IL-8, leucocyte, and granulocyte count in patients with COVID19. This can contribute to an anti-viral immune response [71–73]. In support of this notion, molecular investigation shows that citrullination is mediated by peptidyl arginine deiminases (PADIs) in response to an increase in the intracellular calcium level, thus leading to the chromatin condensation. However, excessive

Netosis are associated with an exaggerated immune response, which may be harmful to the host. Such mechanisms are needed to be explored in detail.

In addition to the post-translational histone modification, RNA modifications such as m6A modifications are highly instrumental in exerting anti-viral immune function through cellular metabolism rewiring [3, 74, 83]. m6A modifications are found to be highly conserved in different members of Flaviviridae, including dengue, Zika, and West Nile virus. The hepatitis C virus (HCV) study shows that m6A machinery negatively regulates viral infection by interfering with the packaging of viral particles. Methylome analysis of host and SARS-CoV-2 shows that infection with SARS-CoV2 is associated with the relocation of crucial m6A regulator from the nucleus to the cytoplasm and plays a prominent role in host immunity [75]. Furthermore, SARS-CoV2 protein 3b can interact with host protein machinery like RUNX1b in an ERK-dependent manner and actively participate in T cell differentiation leading to the chemokine and cytokine response [76]. An infection experiment with SARS-CoV2 in 229E cells shows that p65 mediated chromatin recruitment is associated with inducing NF-kβ signaling, conferring an anti-viral state. Infection with SARS-CoV2 is positively associated with higher transcription factor activity and acetylation of H3 and H4 histone protein [77]. Although the basic pathogenesis underlying SARS-CoV2 infection is clearly understood, the potential epigenetic mechanism governing these events needs to be explored in detail.

6 Epigenetic Basis of Immune Evasion During SARS-CoV2 Infection

Many DNA and RNA viruses fine-tune potential epigenetic marks to safeguard their persistency and latency for a successful evasion from the host immune response. Previously several studies have highlighted the mechanistic basis of immune evasion exerted and adapted by several RNA and DNA viruses [78]. In response to viral infection, functionally important cytokines and chemokines are generated, which are associated with increased antigen presentation leading to the inhibition of viral replication and life cycle.

In addition, the role of ISG (interferon-stimulated genes) in inducing ISG response is shown to be associated with efficient immune function and checks viral infection. Genome-wide DNA methylation analyses have shown altered epigenetic signatures in patients with COVID19 compared to normal individuals, where hypermethylation and hypomethylation of IFN-related genes and inflammatory genes are very precisely regulated by the potential epigenetic player and regulate host immunity [79].

Production of type I IFN is associated with induction of a signaling cascade, causing transcription of several ISG. Evidence from influenza and other RNA viruses shows the role of ISG expression. However, SARS-CoV2 and MERS-CoV were found to delay the significant expression of ISG [80]. It is imperative to note that the

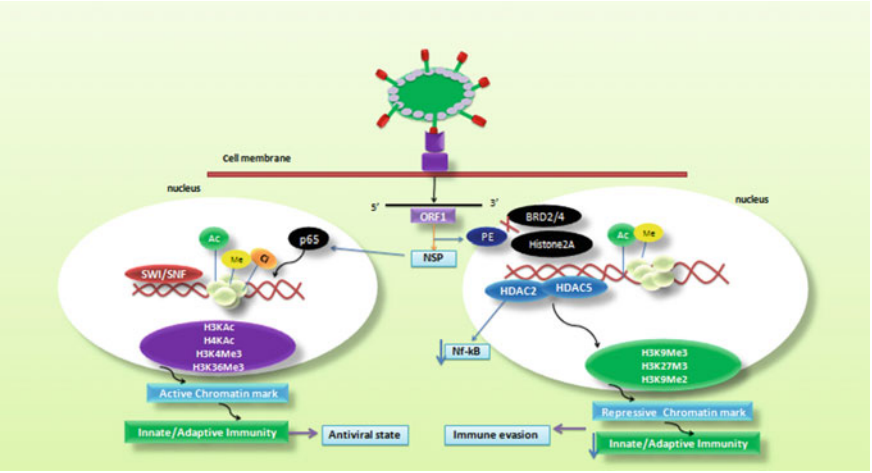

Fig. 1 Implication of chromatin players in conferring anti-viral state and immune evasion

downregulation of ISGs is not due to any alterations in the signaling cascade but due to methylation and acetylation of specific histones induced by the pathogen. SARS-CoV2 induced production of type I and type III IFN in the host leads to induction of histone modulating complex, which renders removal of repressive histone marks like H3K27me3 and puts activating marks like H3K4me3 (Fig. 1).

Histone mimicry was found to play a key role in modulating host immune response. Previously it was observed sequence similarity with histone H3 and C terminal non-structural protein (nsp1) of the H3N2 influenza A subtype. This can lead to suppression of type I interferon response in the host and plays an essential role in viral infection [81]. Furthermore, several lines of evidence have shown the role of SliM (short linear motifs) and IDP (intrinsically disordered proteins) in mediating the histone mimicry and host immune evasion supporting viral infection [81–83]. The role of bromodomain (BRD), a conserved structural module of chromatin-associated proteins, and histone acetyltransferases in controlling the transcription of genes have been well studied [84]. It has been observed that BRD can regulate the transcription of genes by interacting with acetylated histones [84]. The role of BRD is instrumental in orchestrating PRR (pattern recognition receptor) signaling and regulation of innate immunity [85]. SARS-CoV2-derived protein E can interact with BRD2, and BRD4, which can alter the activity of BRD histones by mimicking the structure of histones. N terminal of histone 2A exhibits sequence similarity over an alpha helix around 15 residues confined to the transmembrane segment of protein E. This mimicking action of protein E on histone can disrupt its interaction with BRD2 and plays a prominent role in host immune defense [86].

The role of the pathogen-related receptor (PRR) and pathogen-associated molecular pattern, toll-like receptor (TLR), JAK-STAT, and NF-κB in conferring viral

pathogenesis is well known previously [86]. In response to viral infection, induction of TLR and retinoic acids inducible gene-like receptors (RLRs) and nucleic acid sensors recognize the pathogen-associated molecular pattern. Evidence indicates crucial roles of specific accessory proteins encoded by MERS-CoV antagonize NF-κB signaling to evade the host defense [87]. However, the viral origin of different proteins and proteases is associated with the inactivation of these adaptor molecules. It causes silencing of the NF-κB signaling, thus paving the way for immune evasion [88]. Furthermore, evidence from the computational and knowledge-based approach has shown that epigenetic factors like hsa-miR-429, hsa-miR-1286, PRDM1, and HDAC7 may be associated with the modulation of several immune signaling pathways and develops an efficient immune evasion strategy [89]. Although immune evasion plays an instrumental role in mediating the viral pathogenicity, the potential mechanism governing the epigenetic basis of SARS-CoV-2 immune evasion in the host needs to be explored.

7 Future Perspective and Conclusion

The role of epigenetics is of remarkable importance in the arms race between host and virus. Manipulation of host epigenetic modifications plays a key role in the evasion of the pathogen from the host defense mechanism meant for survival and successful infection strategy adapted by viruses. The virus itself has evolved its structural and accessory proteins leading to the modulation of the host transcriptional and epigenetic program crucial for host defense. Infection with SARS-CoV2 is associated with utilizing and exploiting host cellular immune signaling. Although vaccine drive has become a milestone in challenging the immune response, understanding the putative epigenetic events determining T cell and B cell memory development needs to be explored in detail. They understand the potential interplay between viruses and host, responsible for viral latency, the localization of virus to specific chromatin sites, the recognition of potent epigenetic signature as a consequence of association with stages of infection, and the chromatin basis of immune regulatory genes. Given the crucial role of epigenetic mechanism, it is exceedingly important to consider that epigenetic machinery is the potential target to regulate the SARS-CoV2 mediated associated mortality and morbidity. Currently, only a few clinical trials are in progress (NCT04403386, NCT04411563) to decipher the epigenetic aspect of SARS-CoV2. However, more clinical trials should be aimed to explore the mechanistic underpinning of epigenetic events determining anti-viral and immune evasion mechanisms. Past years have significantly contributed to understanding chromatin modifications associated with host–pathogen interaction. Although the significant role of potential epigenetic modulators in cancer and other diseases has been elucidated well (Table 1), how these drugs affect the mechanistic underpinning during SARS-CoV2 mediated infection is poorly understood. Therefore, more studies in the clinical setting are required for better precision and anticipatory medicine. A remarkable development in the single cell-based chromatin analysis approach; microscopy-mediated

single-molecule real-time imaging strategy for chromatin dynamics, epigenome microarray, chromatin immune precipitation with next-generation sequencing, and FISH approach will be an innovative approach to understanding the complex dynamic cross-talk between host and virus interaction. It is imperative to consider that epigenetic drugs targeting SARS-CoV2 in the clinical setting are lacking. Therefore, more future studies should focus on understanding and elucidating the novel paradigm involved between SARS-CoV2 and the host.

Table 1 Therapeutic implications of epigenetic drugs against SARS-CoV2 infection

Epigenetic drug	Host immune target	Mechanism of action	Clinical trials reported
Decitabine [5-aza-2-deoxycytidine (5-azadC)]—(nucleoside-based DNMT inhibitor)	Macrophages	Inhibition of DNA methylation in macrophages; suppressing inflammation and IFN response	For COVID-19 pneumonia—ARDS treatment (CTI: NCT04482621)
Azacitidine	DNA MethylTransferase 1 (DNMT1) inhibitor	Inhibition of the DNMT1 enzyme and thereby may be used for controlling coronavirus infections	N/A
Anacardic acid	Histone AcetylTransferase 1 (HAT1) inhibitor	Inhibition of the HAT1	N/A
Vorinostat or suberanilohydroxamic acid (SAHA)	Histone deacetylase (HDAC) inhibitor	Can be investigated for their potential in interfering with the non-structural proteins governing the viral life cycle in host	N/A
Panobinostatan belinostat	Epigenetic enzyme histone deacetylase (HDAC) inhibitor	Non-structural proteins viral life cycle in host	N/A
Romidepsin	Epigenetic enzyme histone DeAcetylase (HDAC) Inhibitor	Non-structural proteins viral life cycle in host	N/A

(continued)

Table 1 (continued)

Epigenetic drug	Host immune target	Mechanism of action	Clinical trials reported
Valproic acid	Epigenetic enzyme histone DeAcetylase 2 (HDAC2) inhibitor	Inhibition of the HDAC2 enzyme and thereby may be used for controlling coronavirus infections	N/A
Curcumin—natural compound	ACE2 gene	Epigenetic silencing of the ACE2 gene and preventing SARS-CoV-2 infection	N/A
8-hydroxyquinolones—natural compound	ACE2 gene	Epigenetic silencing of the ACE2 gene and preventing SARS-CoV-2 infection	N/A
Sulforaphane—natural compound	ACE2 gene	Epigenetic silencing of the ACE2 gene and preventing SARS-CoV-2 infection	N/A

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Epigenetics and Meaning

Body, Meaning, and Time: Healing Response as a Transtemporal and Multimodal Meaning-Making Process

Farzad Goli

Abstract The healing response is not a linear regression to balance. The human organism is an autopoietic system that recreates its balance in forward–backward and multimodal processes. The difference between actual and anticipated bodies generates free-energy, and meaning-making processes emerge to integrate it into the organism's functional closure. The sign systems which form the body and regulate its energy are multimodal, interactive interpretations from the molecular to the inter-subjective levels. The body needs to predict and control energy to keep itself far from entropy. The interoceptive prediction after proprioceptive drift towards an anticipated bodily state is the key to the transtemporal bodily experiences. Many experiments like rubber hand illusion and embodied virtual reality reveal the projective nature of the body and how we can experience other's bodies and other potential bodies. All active or inert treatments have symbolic aspects that figurate a feeling of a relieved body. This essay is about how healing expectation leads to a multimodal image and transient homeostatic interoceptive feelings. We also explore how repetitive experiences of a potential body induce epigenetic changes and form new attractors in the actual body. A nonlocal, semiotic body may integrate our medical knowledge more effectively and unfold new gates to health and happiness.

Keywords Biosemiotics · Mind–body · Free-energy principle · Placebo · Interoception · Embodiment · Healing response

1 Introduction

The focus of this paper is to provide a framework for discussing issues of epigenetics and anticipation from the perspective o biosemiotics. It is my hope that experts in epigenetics and anticipation would care to consider biosemiotics aspects. My own understanding of anticipation was shaped by the works of Mihai Nadin (see

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especially [1]). In respect to epigenetics, I would mention Moshe Szyf and Michael Meany, who are credited with establishing the field of behavioral epigenetics.

2 A Healing Journey in Time

> The action of the soul on the body is the action of one part of the body on another.
>
> Diderot, *Éléments de physiologie*. 1774, p. 333

The human brain is a spaceship, and the healing response is indeed a mysterious journey in time. The familiar treatment pictures show us how chemo-physical agents restore the disordered processes to the order and re-establishes homeostasis. It is a flash-forward story from an ill-present body to a healed-future one. Nevertheless, the true story is a much more complex adventure pending in time.

Our energy and consciousness wander between no time and time and past-future and present. Mind, as Luhmann [2] explains, works by a binary time code of past-future, and now is a supposed border in between "no more" and "not yet." While the events and wants are symbolized in the autobiographic memory as memories and predictions, our present body reflects them by various patterns of readiness to act [3, 4]. So, to have a timely body that reacts appropriately to the current perceptions, we need to go beyond the "duration" experience to the "nowness" of bodily awareness [5]. Thus, the body is possessed by the moment-to-moment time of experiencing self and the durational time of narrating self [6]. The body is always a present being, moving in the path of the winds of times.

Varela [7], pursuing Husserl's views on temporality [8], assumes moments of nowness embedded in broader temporal contexts in terms of retention and protention. Retention refers to the temporally backward-extended present, consisting of a tail of past events. Protention, in turn, refers to the anticipation of the next moment implied by nowness [9]. Therefore, past and future can be defined as the extensions of narrative nowness, the symbolic extensions of the lived body. We feel our inner body about our remembered or perceived objects and orient towards/away from them. The brain, as an "interoception" predictor machine, modifies our "proprioception" and our orientation towards related objects based on prior knowledge and regarding the "exteroception" context [10]. The brain actively predicts and searches for the more likely cues to the more synchronized interoceptive states. The mirror neurons reflect intercorporeal images of the others as well as the transtemporal image of our remembered and anticipated bodies [11]. Such a multi-time body is more like a live holographic image that reflects various times-modes-persons in its presence. I think it's now more tangible why a bodily process like healing is not a linear flash-forward story.

From this viewpoint, a therapeutic intervention before everything is a supposed-to-heal agent which associates homeostatic interoceptive state memories. According to Lieberman et al. [12], activation of the right ventrolateral prefrontal cortex (rVLPFC) was related to the positive expectations about the treatment (i.e., "I believe I am

going to be less bothered by pain now"). These interoceptive inferences rely on the somatic and symbolic memories, evoke linked psychoneuroimmunological processes [13]. Bodily memories of the attachment style and conditioning learning, encoded in our implicit and autobiographical memories and extended to the anticipations, can fundamentally change the healing responses [14]. Several types of conditioning modify the psychophysical responses to all forms of therapeutic interventions, from classical to operant and from abstract to immune conditionings. That's why healing responses can arise before pharmacodynamic interactions and even without them [15]. This complex Mind–body kinetics is known as the placebo response. Placebos reveal the psychophysical regulatory power of anticipation [16], attachment [17], and clinical relationship [18]. They disclose the healed-body-image which is embedded in all types of active and inert therapeutic agents [18]. Many studies show interoceptive imagery can change the postural and physiological factors [19, 20]. Each body in itself has, or can have, a dynamic, healthy version of the whole body that can be activated and developed by various energetic, material, symbolic, and reflective signs [21].

The homeostasis concept implies such a genetic blueprint of a healthy organism and illustrates a retrospective healing process towards re-balancing and re-covering [22]. In contrast, autopoiesis's more sophisticated c shows a prospective balance and reflects the co-emergence of salutogenesis and healing responses in a complex environment [23]. The autopoietic procedure of healing response is a back-and-forth current, back to the functional closure and forth to the structural openness. Therefore, the body is always on a back to the future journey; it tries to find sameness and will find itself in some otherness.

In this chapter, I want to shed light on placebo response as co-emergence of the actual and potential bodies' interactions and how temporal neural networks are epigenetically led to new adaptive attractors [24]. To explore such a nonlinear and multimodal process, we need first a metalanguage to integrate our findings on the symbolic and physiological aspects of the healing response. Biosemiotics and free energy principles are the explanatory models I use to trace energy-information flows through the biopsychosocial changes in the healing process.

3 Meaning and Energy: The Brain as a Bitcoin Machine

The potential difference between anticipated and felt bodies arises from energy and motivation, and information to organize free energy. According to Gregory Bateson [25, pp. 1–18], information is "a difference that makes a difference;" he elaborates on the concept that "Difference, being of the nature of the relationship, is not located in time or space." Information, or from an upward-down view meaning [26], is the fundamental property of nature from the biological and the symbolic level of mind to the informatic level [27]. As Luhmann [28] defines it, meaning is the continuous processing of the difference between actuality and potentiality. Of course, for Luhmann, meaning assumes a central position for any social and psychic systems

[29] and not the biological systems. Barbieri [30] follows the Peircian theory of signs [31] and adopts a synechistic approach to meaning and nature:

> If we look at the evidence of life without the preconditions of the present paradigm, we discover that semiosis is there, in every single cell, and that it has been there since the very beginning. This is what biosemiotics is really about. It is not a philosophy. It is a new scientific paradigm that is rigorously based on experimental facts. Biosemiotics claims that the genetic code (1) is a real code and (2) has been the first of a long series of organic codes that have shaped the history of life on our planet.

Thus, differences in various levels of the organization rise between the "sameness" of self-referentiality and the "otherness" of structural openness of any living system. The brain works as an information or difference engine to survive by acquiring new high-grade or free energy supplies to maintain an internal state far from entropy [32, 33]. In other words, the brain aims to maximize mutual information and complexity of meaning-making systems to minimize the free energy that arises from the differences between its expectations and ever-changing perceptions. Like a bitcoin miner, the brain transforms energy into the network states. The brain puzzles emerge under the pressure of differences in anticipation vs. reality. The more value is the more amount of information in its Wienerian sense, the measure of the degree of organization [34]. The more integrated body, coherent narrative, synergetic relationship, and unconditional intentionality can be mentioned as the aspects of the higher amount o or the more cohesive meaning systems [35, 36].

In this sense, healing, as a meaning-making process, is the continuous processing of free energy between the actual body and the potential body to maximize value-creating and farther state from dis-order. It is a meaning processing towards wholeness and totality of functions. Healing and health have the same etymological root as the word "whole" [37]. It may imply such a definition of health as the integrity of parts and functioning as a whole, and healing is the way back to this state.

When we use a supposed-to-heal agent, a potential healed body has already been formed and felt like a layer in our actual body [12]. Because the anticipated body is felt temporally. We feel a possible state of the future in our present body. Imagining potential future situations and states the self may encounter [38] and self-relevance biases in memory encoding [39]—all involve positioning the body in a better context. In other words, a healed body has been figured in our ill body; pleasant and painful interoceptive states are paging alternately through the body. Any supposed-to-heal factors can evoke the symbolic and somatic memories of the enjoyable state. The default mode network and fronto-insular cortex bridge interoceptive awareness and autobiographical memory [40, 41, 43, 44]. This enables projections about future events and alternative courses of action by imagining their impact on our overall well-being [45]. So, a potential healed body is narrated, felt, and finally can be created as an actual healed body via a multimodal imagination.

Healing associations of the potential remedy bypass the allostatic body and project prior homeostatic interoceptive experience to the anticipated body image; this transtemporal reprocessing can modify the present Interoception [45]. Repetitive activation of these healing pathways leads to epigenetic changes and emerging the

more adaptive psychoneuroimmunological attractors. So, let's see how a psychosocial sequence of the symbolic signs, composed of verbal suggestions, clinical settings, nonverbal communications [42], and rituals [46], can make a Salutogenic cocktail of opioid, endocannabinoid, serotonergic, dopaminergic, Cholinergic, and oxytocinergic agents [47–49].

4 Meanings and Molecules: How Frog Narrates His Royal Tale!

Where do molecules meet meanings? In what ways can the mind affect the body? What does connect mind and body? Without any hesitation, from a biosemiotic point of view, I want to answer the questions in order; nowhere, no ways, nothing. But why?

Biosemiotics is a metalanguage for studying living systems as the meaning system, which are interconnected in different levels of the organization, from genes to the biosphere [30]. From a folk psychology viewpoint, we have some meanings in our minds in the forms of representations and intentions to treat ourselves and the environment. If the live body can be defined as a flexible meaning system, then there is no need for anything like close encounters of the third kind between the mechanical world of the body and the semantic world of the mind. Cybernetics and semiotics are two complementary ways out of wandering between dualism and reductionism [26]. Our recent studies on epigenetics and psychoneuroimmunology reveal the semiotic nature of the body and how symbols and reflections can be translated to the molecular and vibrational signs and vice versa [21, 50, 51]. The significant and sustainable physiological change due to placebo response and other mind–body interventions like mindfulness disclose the embodied [e.g., 52, 53]. Mind–body interventions can activate temporal neural circuits that are associated with regional metabolic increases involving the prefrontal, anterior cingulate, premotor, parietal, posterior insula, and posterior cingulate and metabolic decreases involving the subgenual cingulate, parahippocampus, and thalamus [54–56]. These temporal changes will likely lead to the formation of new neurological [57], immunological [58], or even intergenerational epigenetic attractors [58].

The symbolic sign systems of mind–body interventions simultaneously provoke the dopaminergic response to anticipation of benefit [48], the oxytocinergic response to feeling attached [49], the analgesic response to activation of opioid pathways [59], affect regulation due to serotonergic modulations [60], synaptic plasticity as a consequence of endocannabinoid rising [47], and anti-inflammatory activities of cholinergic pathways [61]. Placebo, hypnosis, imagery, psychotherapy, and other symbolic interventions can alter the epigenetic code [61, 62].

The biological systems function based on coherent interpretations of sign vehicles and psychological and social systems. Sign vehicles can be vibrations, molecules, cells, words, images, contemplation, and/or social constructs [21]. When you are

laughing while watching a live TV show, your laughter is the endpoint of the series of a wide variety of sign vehicles. Like a relay race, the electromagnetic and mechanical waves of the scene are translated to digital codes, then to the high-frequency radio wave, and then again translated to digital, code and then electromagnetic and mechanical waves on the monitor and speaker, then to alternating electro-chemical translations. Finally, symbolic and reflective sign vehicles evoke and elaborate the meaning of the events, and in comparison, with our prior knowledge, we find them surprising and funny, so you laugh.

Various chains of energetic, material, symbolic, and reflective patterns lead to more complex functions, namely elaborated meanings [63]. The searching-linking (~ Structural openness-Functional closure) nature of life entangles energy and matter and spirals level by level to the symbolic and re-entrant orders [64, 65]. In such a self-referential transcendence stream, who can distinguish bodies from minds?

Bodies and minds are temporal illusions, and the physical and the mental, as Spinoza [66] illustrates, are only two modes or attributes of an implicit/explicit nature. All substances which make the body visible are replaceable, and "life resides in organization, not in substance" [67]. The organization is what forms lived body, and these terms imply the soft and informational meshwork behind the gross body. Thus, the body formally is the mind; the embodied mind is semantically formed and mechano-symbolically acts. From a nondual perspective, we can follow life, health, and illness through the signs' flows or semiosis [68]. Meanings in the forms of physical and/or symbolic dis/functions appear. Different types of signs create different levels of the organization. All three types of signs that Peirce [69] defined can be recognized in human organisms; indexes, icons, and symbols [70].

Indexical signs have a cause-and-effect relationship between representamen and interpretant or between sign vehicle and meaning [69]. All the signs in endosemiosis—the cellular, intercellular, and vital system—are indexes. While in a transitional layer of the body, the image schemas are iconical.

Icons are signs where meaning is based on similarity of appearance [or function] [69]. Image schemata are recurring structures within our cognitive processing that establishes patterns of understanding and reasoning [71]. Connection, up-down, from-to, and balance are some of the image schemata that are indeed abstract forms of daily bodily experiences that metaphorically have been extended to cognitive processes, i.e., bridges between sensorimotor and semantic systems [72]. Many of the traffic and digital icons are from this group of signs.

The most complex and ambiguous form of singing is the symbol with an arbitrary or conventional connection with its referent [69]. Linguistic, mathematical, ritual, and legal signs are samples of the symbolical signs. When we follow the signs beyond the boundaries of genes memes (cultural codes) and from epigenetic and neuroimmune regulation to cognitive-behavioral changes, all can be read on the same page; a complex multilayer text [21, 73].

We can give voice to the lived body and let the body frog transform into a multilingual prince body; a prospective and imaginative body that creates new copies of itself to adapt to anticipatory futures. Interwoven, autopoietic networks of indexical, iconic, and symbolic signs construct a prospective body between habits and

desire, memory and imagination. We need a more complex evolutionary model than Darwinism to realize such a complex system. The "chance" and "selfishness" are mighty forces in the formation of both genetic and memetic codes [74, 75]. In a higher level of organization, we can see the power of "habits" that regulate the gene expression; from a neo-Lamarckian perspective [76, 77]. Yet, there is another evolutionary force, the nurturing force of the whole on its parts which Peirce [78] named "agapism."

The agapistic approach to life, health, and medicine help us to go beyond anthropomorphism bias—which tries to analyze life as the story of the living things—to a dynamic multilevel meaning network (semiosphere) in which beings appear temporally as some complex nodes of the time–space-information meshwork [21, 79, 80]. By seeing through such systemic glasses, an agapistic evolutionary dance of life appears, as Peirce [78] explained. This evolutionary love was superimposed on the worldwide Darwinian wars of the self-referential living systems [81].

The top-down approach of agapism integrates our specialized knowledge about different aspects of human life and provides more fit explanatory models for salutogenesis and healing response. Treatment and pathogenesis are more Darwinian concepts and contain many war metaphors such as disease against body-mind or treatments against disease [82, 83]. The schemata behind healing and salutogenesis are more Peircean and agapistic. As a matter of course, treatment/healing, cure/care, and pathogenesis/salutogenesis can be mentioned as complementary pairs [84]. From this viewpoint, "Salutogenesis" is an epiphenomenon of the self-organizing meaning-making trait of the living systems; "pathogenesis" is misinterpreting conditioning of the systems; "healing" is the systemic tendency to decondition the misinterpreted meaning systems; "treatment" is a deconstruction of the disordered circuits; and "cure" is focused on eliminating dissonant meanings while "care" is the utmost existential mode of consciousness towards being and directed to the whole person.

For effective integration of the healing-care approach into the treatment-cure discourse, we need an integrative evolutionary theory, A Darwinian-Lamarckian-Peircean model of life and medicine. Understanding the genetic, symbiotic, epigenetic, and memetic codes and signs makes our multilingual body more understandable and more manageable in health and illness. After a glance at the semiotic nature of the body, we can go closer to the transtemporal trait of the human body and how the whole body leads energy-information flow to a more fulfilled future.

5 Body: Between Being and Willing

As mentioned before, the difference between expectations and perceptions evokes free energy and an allostatic state of the body. By this force, meaning-making processes emerge to create more coherent relations and a new homeostatic state farther from entropy. In this essay, I use meaning in its vast sense of "the function of a sign in a context." In both phylogenesis and ontogenesis courses, many genetic and epigenetic and symbolic and reflexive signs emerge to minimize the free energy in the

more diverse contexts of space–time-relationship. However, the body as the subject of medicine is still a three-dimensional object, and the body's temporal, symbolic, and reflexive dimensions are mentioned as additional considerations. It is still a hard problem how the body has formed a symbolic and reflexive space for the possibility it its actual order and how the healing expectation leads to psychoneuroimmunological and epigenetic changes [e.g., 85, 86]. We should find psychophysical constructs that transform willingness to the biological functions and vice versa.

Continuous processing of difference and the projective nature of desire, along with evolutionary time, might make abstract and timeless constructs in the human body; image schemas. Johnson ([87], p. xix) defines them as follows:

> For although a given image schema may emerge first as a structure of bodily interactions, it can be figuratively developed and extended as a structure around which meaning is organized at more abstract levels of cognition.

For although a given image schema may emerge first as a structure of bodily interactions, it can be figuratively developed and extended as a structure around which meaning is organized at more abstract levels of cognition.

He explains how image schemata make the possible world of imagination ([87], p. xx). The bodily perceptions such as pressure, resistance, pushing, longing, heaviness, and lightness have been constructed in non-propositional schemas, like from-to, up-down, center-periphery, which make the inference, and prediction possible. Of course, by elaborating these schemas to the symbolic and propositional constructs, this prospective capacity of the body has been developed pervasively [71, 72]. Thus, the indexical and iconical meaning processing evolved into the symbolic meaning system with higher freedom for meaning-making and more predictive capacity [88, 89].

From this perspective, the potentiality vs. actuality can be defined as four-dimensional somatosensory matrices, the different patterns of bodily openness and orientation towards a likely vs. felt bodily state. Our somatic and symbolic memories from forces and barriers shape these openness-orientation matrices. Johnson [71, p. 53] states: "In particular, we understand mental reasoning processes involving forces and barriers analogous to physical and social forces and obstacles." The metaphorical translation of these somatic schemas may be elaborated to the reasoning and the intentional states like emotions [90].

A supposed-to-heal intervention evokes these somatic matrices toward a more integrated bodily state. All the associated molecular, schematic, and symbolic meaning-making processes activate and may lead to new Salutogenic attractors. The body gradually constructs new pathways of meaningfulness that can be assessed by the degrees of coherence of elements within a whole system and conformity of expectations and observations [91]. The co-emerged meaning-making systems bridge the gap between the expectations (~ needs/desire/potentiality) and the observations (~ resources/barriers/actuality) [28, 36]. Meaning-making is how living systems re/construct themselves, assimilate environmental signals in their functional closure, accommodate themselves to the environment, and create their lifeworlds (Umwelt) [92, pp. 107–110] [93]. Memory induces degrees and forms of expectation in every

Fig. 1 Co-construction of memories and anticipation in the context of perception (Adapted from the "*Drawing hands*" by Maurits Escher, 1948)

given perceptual context, and the openness and orientation of each expectation may change our memory and belief system in the context of the present perception. The lived past has inertia to continue itself and be our future, and the imagined future alters the structure and function of the remembering past; all these happen in our present lived body. It is something like the illusionary painting of Maurits Escher, where two hands make the other simultaneously (Fig. 1).

Meaning, in this sense, is re/interpretation of prediction and minimizing the errors forced by free energy, which arises from the Potential difference between anticipation of a relieved body and perception of a painful body. The brain is an intentional system that actively predicts interoceptive state in the interactive contexts of memory and exteroception. It frames the proprioception, the way we approach the objects and future [10]. All these dimensions of perception are reframing the provocation of their errors. Phenomenologically, we call here Interoceptive errors "howness," exteroception errors "whatness," and proprioception errors "towardness." These error signals turn on the meaning-making machines to assimilate free energy. The multimodal appraisal of a distressed body (howness) is attributed to the barriers and resources (whatness) and oriented to a released body in a more attuned situation (towardness). Ashar et al. [45] explain the role of the appraisal networks in the formation of a whole healed body:

> This network is involved in emotion generation, social and self-referential cognition, and value-based learning and decision making, pointing to a common core function of flexible, conceptual, and affective thought. This system allows us to simulate potential outcomes

and develop expectations about future events. It also allows us to relate those events to a representation of the self, including our broader goals and overall well-being.

So, potential outcomes in the form of a whole-body image are imagined, felt, and translated to new epigenetic and functional pathways. The human organism needs to recreate its biopsychosocial coherency by changing energy-information flows via autopoietic co-emergence of a new meaning system [94, 95]. Signal errors ($\bar{e}$) are the guide angles of this complex system. Our autobiographic and somatic memories are symbolized in the form of narrative and body image; these memories and habits tend to prolong in the form of expectations, while our inward/outward perceptual windows may have a different multimodal representation, leading us to change our openness and orientation (see Fig. 2). Any supposed-to-heal agent can change the expectation and narrative; altering what we attribute to our bodies and where we are oriented changes how we feel our bodies.

The interception errors (homeostatic feedbacks) are at the core of body semiosis [96]. Howness is the organism's intrinsic value and attributes to the objects of exteroception and memory (whatness) and shapes our towardness in our proprioceptive figurations [97]. Howness, whatness, and towardness are comparable with the three dimensions of Dasein's being-in-the-world that Heidegger [98] defines; attunement, discourse, and for-the-sake-of. Prediction error signs are circularly being reinterpreted and changing our qualia, openness, and orientation to minimize free energy and reduce tensions and dis-orders. Many prior codes like placebome [59], memes [99], as well as symbolic significance of social contexts [100, 101], and interoceptive awareness and openness [102], can modify the multimodal perceptual errors and facilitate the healing response.

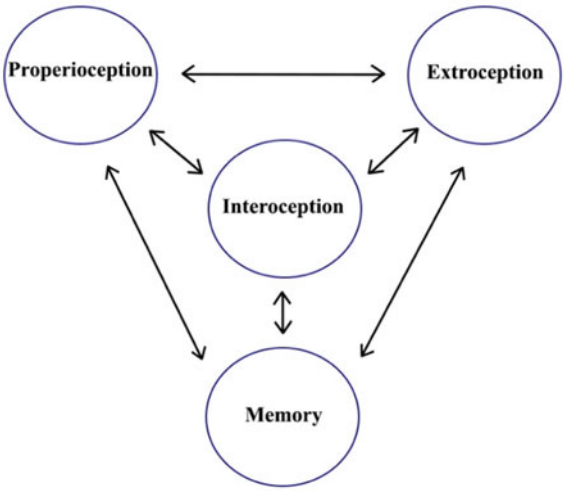

Fig. 2 The dimensions of bodily perception

6 From Local Body to Temporal Bodies

Where is the boundary of my body? Is the limit of a body drawn at the skin? What about social proxemics? And the ownership field? Where do the body finish and the environment start? Are there any symbolic or imaginary bodily extensions beyond the epidermis? When we are driving a car or ship; when we cry while watching TV; When our avatar overcomes its fears in virtual reality, even whenever we feel better just after getting a prescription or consuming a placebo, we are something beyond our local body. Our embodied boundaries are ever-changing in different perceptual and/or imaginary contexts.

We experience altered bodily states when the vehicle, the movie personage, or virtual or future bodies are subject to change. The body not only inhabits but also produces space [and virtual bodies] [103]. Many experiments like the rubber hand illusion and the interoceptive drift from the actual hand to the rubber hand show the fluidity of bodily sensations and temporality of body ownership [104]. Embodied virtual reality experiences are another phenomena that reveal the nonlocal aspects of the human body [105].

The interoceptive inference after proprioceptive drift towards an anticipated bodily state is the key to the transtemporal and nonlocal bodily experiences [106, 107]. When we contemplate the projective and prospective bodily phenomena, we convince to elaborate our local body image to a body with temporal limits [108, 109]. Even before rising the posthuman problems of the metaverse [110], cyborgs [111], and cloning [112], the body was wandering in time and potentiality. Beyond the being inhabitant of time, the body is the lived time, and time is the body [113].

Our mirror neurons and prior knowledge transform perceived bodies into our bodily perceptions and potential bodies into actual feelings [11, 114]. Our body can figurate anything, anywhere, and anytime we attend is not what the objects are and not necessarily what is perceived. In a phenomenological mirror, the body boundaries look more shapeless than an amoeba, and its extensions and arrays change not only in space but also in time. Self vs. other and actuality vs. potentiality differences run meaning-making systems to integrate free energy into organism functional closure. This is the autopoietic way of recreating the sense of coherence and healing response [115, 116].

First-order observations are initially reflective and make us more or less aware of our actual, interoceptive energy flows. Howness is what we can experience immediately; the quality of being-in-the-world. The first-order observations are associated with our prior knowledge and first-order expectations. The differences between self-observations and self-expectations activate the primary meaning-making processes. Furthermore, the lived body is open and exposed to the other bodies and sees and explores itself through the others' mirrors from the beginning stages of development [117]. Thus, the intrapersonal meanings are communicated in the socio-cybernetic fields and interpreted by others (see Fig. 3). The second-order observations

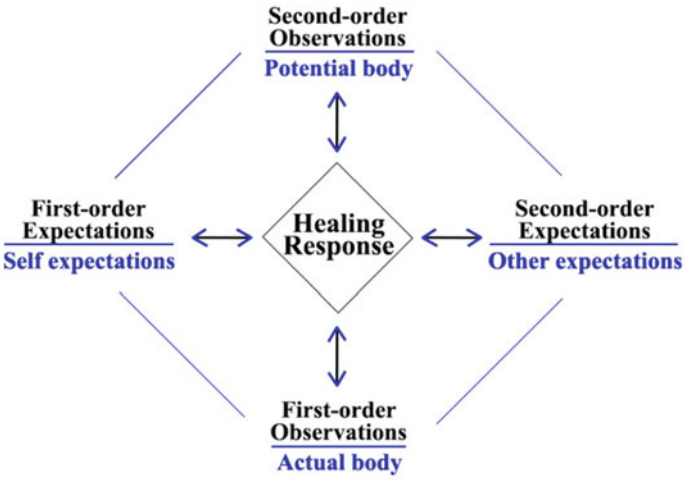

Figure 2: Healing response as a result of the complexdynamics of actual-potential bodies and self-other's expectation

Fig. 3 The healing response and complex dynamism of actual-potential bodies and self-other expectations

and expectations reinterpret the primary interpretations in emergent intercorporeal-intersubjective systems [118, 119]. The second-order observations are usually introjected as figures of bodies we could be. Foreseeing and mindsight both emerged from mirror-neuron function [11]. Some of the other's observations and expectations are projected to our future bodies. In different sociosomatic contexts, these projections lead to various symbolic and epigenetic attractors [101, 120]. That's why the meaning response to the supposed-to-heal agents is variable from one personality trait to the other [121], from culture to culture [122], and from one doctor-patient relationship to the other type [123]. The lived body is not a local object but is an intercorporeal, intersubjective, and transtemporal being.

From a biosemiotic perspective, information is medicine, and medicine is information. Any energetic and material intervention induces chemo-physical changes interpreted by cells, CNS, and other subjects. Any symbolic and reflective interventions or alterations are also interpreted intersubjectively as well as neurologically, cellularly, genetically, and epigenetically. For example, when a person has colectomy, this mechanical intervention and all the metabolic and microbial consequences are integrated into the other organs, and new meaning/functions are formed. Furthermore, some higher-order meaning-making processes run and construct new body image, illness beliefs and behavior, and familial and social support systems. Thus, biomedical interventions are also informational and semantic agents. On the other hand, semantic interventions and soft realities such as placebos, nocebos, doctor-patient relationships, mindfulness, and psychotherapy can be mentioned as epigenetic medicines [62].

The symbolic and reflective signs are more ambiguous and interpretable than the energetic and material signs, but we can mention them as higher order bodily meaning systems. The semiosis of an active or inert therapeutic agent is traceable from inter corporeal/intersubjective context of intervention to the psychoneuroimmune enhancements until epigenetic changes expressions.

7 The Healing Response Formula

The biosemiotic metalanguage connects the soft reality of imaginations and intentions to the hard reality of stimuli and behaviors. The healing response is always something more than a biological self-regulatory response or regression to the mean or other natural history effects [52]. Our investigation in this essay focused on meaning response or actual placebo response, which varies from context to context. We can summarize the main factors that evolved in a perceived healing response as follows (Fig. 4):

Perceived healing response represents what we experience in the healing procedure. Thus, before everything, we should mention the gap between perceived and actual healing; the cognitive-statistical errors. Regression to the mean [124], and patient recall errors [125] are the most well-known biases that are confused with the meaning response [59].

Contextual factors change our mind–body functions independent of the semantic and chemo-physical interventions, such as the natural history of the disease, personal history of the patient (e.g., attachment style, personality, belief system, psychological

$$\mathbf{HR_p} = \mathbf{E_{cs}} + \mathbf{C_x} + \mathbf{M_n} + \mathbf{B_a} + \mathbf{V_r}$$

$$E_{cs} = RTM + PRE$$

$$C_x = NH + PH + HE$$

$$M_n = PR - NR$$

HR_p: Perceived Healing Response, **E_{cs}**: Cognitive-statistical Errors, **RTM**: Regression to the mean, **PRE**: Patient Recall Errors,

C_x: Contextual factors, **NH**: Natural history of the disease, **PH**: Personal history, **HE**: Hawthorne effect, **M_n**: Meaning induced responses, **PR**: Placebo response, **NR**: Nocebo response,

B_a: Bodily awareness, **V_r**: Verum effects,

Fig. 4 Perceived healing response elements

capital, coping strategies, lifestyle, random factors). The Hawthorne effect is the primary effect of second-order observation.

The meaning response, used here in its conventional sense, implies the meaning response to the symbolic signs of intervention and related communicative elements [60, 123]. The meaning response comprises many interactive factors, such as doctor-patient relationship communication, memes and discourses, and all forms of related verbal and non-verbal suggestions [21]. While placebos represent expected desired responses, nocebos indicate expected unpleasant responses directed against the sense of coherence [126]. Bodily awareness re-entrant flow of our own bodily inner and outer perceptions. Bodily awareness is non-judgmental and keeps us connected to the present moment and our bodily ownership and agency [60, 127]. Bodily awareness is a reflective semiosis. It can be translated to the symbolic signs and evaluated. Still, when we experience our whole body as an integral and non-judgmental state of mind and a more secure emotional state, it may facilitate optimistic visions, higher self-efficacy, and a more positive feeling about our future body. Interoceptive awareness and openness are predictive factors in meaning response [102]. As we mentioned before, all these elements can be analyzed as biosemiotic processes that make meaning/ functions via indexical, iconical, and symbolic sign systems, while the verum effect is the scene for the indexical signs (energetic, biochemical, and mechanical interventions). Bodily awareness is the primary realm of reflective signs. The reflective signs are pauses or re-entrances in the current of signs, like silence in music that makes qualia and meaning in the context of signs. The meaning response is the symbolic functioning representing meaning responses of signs through different layers of a healing response. A nonlocal, semiotic body may integrate our medical knowledge more effectively and unfold new gates to health and happiness.

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To Know: The Intersection Between Anticipatory Action and Epigenetic Processes. God, Science and the Last Question

Elvira Nadin

Abstract Epigenetics triggers genetic processes in the living. Anticipatory processes pertain to the entirety of life. Understanding their relation is a prerequisite for approaching spiritual aspects of individual and social life. Epigenetic processes associated with non-material promoters are part of the encompassing anticipatory action through which life is preserved. Ideas can be as influential as substances (food, natural poisons, synthetic interventions such as pharmaceuticals or genetic engineering). Empirical evidence of anticipatory expression suggests that epigenetic processes are, by necessity, grounded in the anticipatory nature of life. Based on these considerations, we advance here the hypothesis that what explains the role of religion is the human drive to know.

Keywords Anticipation · Epigenetics · Genome · Religion · Spirituality

1 Preliminaries

To establish a common understanding of the concepts involved, especially when it comes to religion, is indispensable. In the spirit of C. S. Peirce's Ethics of Terminology [1], working definitions are spelled here out as a preliminary. They serve as the "common denominator" for assessing data and for the interpretation of their meaning.

Anticipation: the current state of an anticipatory system depends not only on the past state (or states), but also on possible future states. It is not prediction, forecast, expectation, guessing, conjecture.[1] It is always expressed in action [2].

[1] Nota bene: Unfortunately, dictionary definitions and those peddled on Wikipedia are frozen in a time when little was known about the role of anticipation in living processes. What was known trickled down from the less than precise formulations within psychology. Even in our days, the discourse on anticipation remains rather unfocused.

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Epigenetics: the study of changes in organisms prompted by modification of gene expression rather than alteration of the genetic code itself (Oxford Languages Dictionary).

Genetics: the scientific study of genes and heredity—of how certain qualities or traits are passed from parents to offspring as a result of changes in DNA sequence (National Institute of General Medical Sciences). This definition is questioned by scientists seeking a better understanding of reproduction in the living [3].[2]

Matter: that which occupies space and possesses rest mass, especially as distinct from energy. This is a physics informed definition. Alternative definition: substance from which everything is made, but distinct from mind and spirit.

Religion: the belief in and worship of a superhuman controlling power, especially a God or gods; a particular system of faith and worship; a pursuit or interest to which supreme importance is ascribed (Oxford Languages Dictionary).

Religiosity: the quality or state of being religious; religious feeling or devotion (Merriam Webster).

Ritual: spontaneous or staged ceremony consisting of actions performed according to a prescribed order (Oxford Lexicon).

The spiritual: relating to or affecting the human spirit or soul as opposed to material or physical things. Sometimes defined as: relating to religion or religious belief.

Spirituality: the broad concept of a belief in something beyond the self. It may involve religious traditions centering on the belief in a higher power, but it can also involve a holistic belief in an individual connection to others and to the world as a whole [40].

These definitions are not normative. They suggest a shared meaning for the purpose of speaking/thinking about the same things. Evidently, there is no consensus on definitions, as there is no consensus on the science on which they are based.

Although the role of ideas, ranging from philosophy to science, aesthetic expression, and motoric activity will be alluded to, the focus is on what is broadly defined as religion or religions (views and practices). They are neither justified nor subjected here to critical evaluation. The role they played during humankind's history changed drastically over time, often because science proposed itself as a more effective way of questioning reality, and of facilitating change. But even in our days, religion, in a variety almost impossible to account for, remains a factor impossible to ignore. Its role in community life, in politics, in promoting or justifying adversity that might lead to human suffering and death cannot be properly assessed.

[2] This definition is questioned by scientists seeking a better understanding of reproduction in the living [3].

2 Anticipation

Between prolepsis and *antecapere*, i.e., notions reflecting awareness of foresight, and the current use of the term anticipation, there is a documented history of how they were defined and how they affected human consciousness of change. The term *prolepsis*, (cf. Merriam-Webster) means "the representation or assumption of a future act or development as if presently existing or accomplished." The term is from the Greek, meaning "the process of taking in front of" or "anticipating." *Antecapere*, from the Latin suggests taking possession (not only of things) beforehand. Other notions can be added. They reflect awareness of foresight, and, more recently, focus on actions ahead of danger, and the use of "positive" cues from the environment. Empirical observations regarding how future possibilities informed actions in organisms have led to a record that suggests that reproduction, preservation of life, and adaptability imply anticipatory action. Only after progress was made in the scientific inquiry of what defines life [4] did it become possible to gain a new perspective of living processes. Once creative aspects of human activity become the subject of inquiry within physiology, cognitive and neurosciences, and of evolutionary biology, it became possible to identify the role of preparation [5] in various motoric and cerebral activity (sports, dance, playing instruments, etc.). Interdisciplinarity is not optional in studying anticipation.

With this in mind, a science of anticipatory processes has by necessity many originating authors, coming from various disciplines. These were identified [6] and further studied. In particular, the role of Soviet/Russian scientists (Bernstein, Beritashvili, Ukhtomsky, Anokhin, and Uznadze) in the 1920s to 1950s must be highlighted [7]. In his work focused on aesthetic creation, Nadin [8] suggested that creativity is by necessity the outcome of anticipation-supported expression. His book, *Mind—Anticipation and Chaos* [9], placed anticipation in the perspective of dynamic systems. Rosen [10] was the first to dedicate a whole volume to the subject.

In dialog with Rosen (aware of Nadin's book mentioned above), Nadin broadened his own inquiry, and engaged a large number of researchers in an exchange of ideas, hypotheses, and methods of inquiry. Important publications and conferences, organized by the Institute for Research in Anticipatory Systems, were dedicated to the subject: *Time and Conscious Brain*, 2011; *Anticipation—Examples of Anticipatory Expression in the Framework of Neuroscience*, 2012; *Anticipation applied to information technology, neural networks, education, politics, biological systems, engineering*, 2014; *Anticipation: The Interdisciplinary Perspective*, 2015; *Anticipation in Medicine* 2015). In 2005, Nadin conceived and built the AnticipationScope™ as an attempt to quantify aspects of anticipatory expression. The multi-year Seneludens project (https://seneludens.utdallas.edu/) addressed the challenges of aging, especially decline in anticipation, by stimulating plasticity (understood in a broad sense.) Experiments in which the AnticipationScope[3] was deployed eventually engaged 170

[3] The AnticipationScope is reminiscent of the cyclogrammerty method that N. A. Bernstein developed in 1921–1925 while working at the Central Institute for Labor. Bernstein registered movement kinematics and showed that the joints involved interacted, correcting each other.

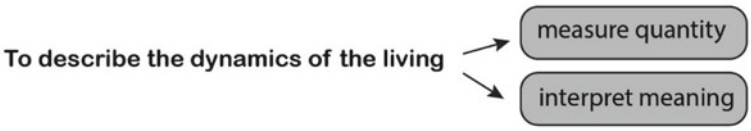

Fig. 1 Anticipation underlies change in the living. The AnticipationScope opens access to measurement data and to meaning interpretation

subjects, and representative in terms of gender, age (6–94 years), race, and cultural background. Data were accumulated in an integrated digital environment set up to capture motoric expression, as well as to document cognitive aspects involved in an *Anticipatory Profile* for each participant. As the experiments advanced, it became clear that in describing motion, the data was significant as the repository of knowledge acquisition through motoric expression, but also of intricate adaptive processes [11–13]. Most important was the realization that anticipatory action affects performance, understood in the broadest sense of the word.

Anticipatory action pre-dates physical performance (such as in sports), cognitive and aesthetic activities (playing instruments, for example), learning. In order to describe the anticipatory nature of preparing for and executing activities (running, playing the piano, golf, washing dishes, sitting on a chair, driving, etc.) a digital "film" of the activity was generated. It integrates motion capture data and sensor data pertinent to the physiology and the cognitive processes involved. While data are important and continue to be processed currently using AI-based analytic tools, the focus of the research was on meaning (Fig. 1).

The research of N. A. Bernstein (On the Construction of Movement, 1947/2020) was experimentally tested and empirically confirmed in the antÉ lab. Moreover, the investigation highlighted aspects that escaped the attempts at quantification of scientists in the last century. In this process, attention shifted from what N. A. Bernstein called dynamics, and even from the role of the central nervous system (which was his focus). The various functions of anticipatory processes in the "to know" how—knowing how to perform an action—were made explicit in particular applications. "*Anticipation and Performance, AnticipationScope and the Anticipatory Profile, with application to the game of golf*" (in collaboration with Eben Dennis, a golfing professional and instructor, 2013, Fig. 2), is a project developed by Robert W. Fuentes, assisted by several graduate and undergraduate students, developed (for the competition "Inventing the Future," 2011).

Others examined the role of movement in therapy: Amazing Grace: African dance movements for the maintenance of fitness and anticipatory functions in the aging (in the framework of the Seneludens project). Germaine Acogny, "The mother of African dance," developed an elaborate routine inspired by exercises (dance movements and singing) in a village in Senegal. Stimulating plasticity was the implicit goal. It turns out that brain plasticity and genetic plasticity are connected. Acogny's program was performed during her visit to the Institute and recorded for research purposes. It

Fig. 2 Quantifying anticipatory aspects of golf playing

forms a repository of a performance that testifies to a culture of rich traditions, in which the physical and the spiritual are intertwined.

Anticipation-driven *Adaptive Architecture Assisted Living* (under the guidance of Asma Naz, at that time a doctoral student at the University of Texas at Dallas, 2013–2018) revealed new perspectives on how behavior is an expression of knowledge, uniting past (experience), present, and the possible future [39]. Interacting with Duke University DiVE, the Institute acquired data on the effects of various parameters (e.g., color, texture, lighting, size) for a living-working space through an immersive virtual environment.

Interaction with Professor Hubert R. Dinse [14] of the University of Bochum (Neuroinformatic Institute) led us to further investigate brain processes involved in anticipatory action. In particular, plasticity—not only of the brain—became the focus. Such expressions of anticipation are necessary for survival. They can be maintained and regained even after brain performance diminishes with aging or is affected by trauma. Testing and validating the effects of interactive games for maintaining motoric and cognitive health in the aging (in cooperation with C. C. Young Community Centers, Dallas TX, and Xavix, Inc., San Diego CA/Shinsedai, Inc., Japan) afforded additional knowledge regarding the unity between the physical, the cognitive and the spiritual.

All these experiments had clearly identified targets (represented by measurable behavioral performance), but also "soft" targets. Indeed, the fully anonymized database of the persons participating in testing anticipatory performance was meant to contribute to the Anticipatory Profile of each individual. The relatively common identifiers (age, gender) were complemented by self-defined characteristics (medical history, medication record, social-economic group, sexual orientation, religion, political affiliation). The premise is straightforward: anticipation action reflects the holistic nature of living processes. They are not reducible to the organism's make-up (molecular inventory), to genetic expression, or to brain-controlled activity. The integration of background data and of interactively generated data was, and still is, an ambitious undertaking. It does not suffice to take note of the fact that reductionist-deterministic explanations are incomplete; one must try to break the limitations inherent in closed-system experiments. The ideal of producing a wearable AnticipationScope that would be like a "skin," (Fig. 3) includes research into areas of extreme anticipation expression (e.g., high-performance sports, air traffic control, military maneuvers emergency situations).

Fig. 3 The AnticipationScope in various configurations: motion capture, mobile, full body wearable

3 God is in the Question Mark

Properly deployed, the AnticipationScope could also account for the specific role of factors such as beliefs (including religion) in human performance. Just to frame the question let us consider some hypotheticals:

1. Is there any relation between human performance (no matter in which field) and beliefs? Formulated this way, this question is larger than belief in God or gods, or the role of religion in an individual's life.
2. Data (from brain activity research, as well as from genetics) concerning the role of belief (religious or otherwise) comes in different formats. Assuming that the heterogenous data can be processed in a uniform manner, can inferences be made across disciplines?

Of course, hypotheticals return at most more hypotheses, i.e., suggestions for more empirical evidence. Moreover, while a piano player or gymnast might identify with some belief (e.g., "I am a Christian" or "I am an atheist"), it would be speculative to infer from a rather undefined entity (gods, atheism, or even agnostic scientific beliefs) to the success or failure of a performance. Moreover, although Bach's music, for example, was composed for the Church, it is appreciated by believers (of various religions) as well as non-believers. Cathedrals, synagogues, mosques, monasteries, Buddhist temples, etc. are visited for their historic relevance, not necessarily for their religious messages.

Given this epistemological situation, understanding the role of knowledge in the success or failure of human activity is a path towards assessing the role of belief/beliefs. When physical evidence for an answer is nowhere to be found in nature, humans default to the supernatural. Like in life, those more powerful seem to have advantage over the others. Endowing various aspects of nature with superhuman powers and worshipping them is the extension of submission to the powerful within a community. Religion is born in the reality of human interactions. Not surprisingly many studies have concluded that religion, regardless of its origin, is so persistent that it must be a genetic trait—hence the "God-gene." Let us however focus on what explains its role:

> God was…invented to explain mystery. God is always invented to explain those things that you do not understand […] When you finally discover how something works…you don't need him anymore. [15, pp. 208–209]

Research of anticipatory actions, as a necessary ingredient of what it takes to perform certain activities, and research on brain activity and on genetic aspects of how the living knows the world, can be seen in their unity. Based on very rich data (from experiments in a variety of high-level scientific institutions presented under headings such as *The MRI of religion*, *Brain activity and art perception*, *The cognitive aspects of rituals*, it becomes possible to identify links to the genetic profile of individuals, as well as to heir Anticipatory Profile. The particular objective is to differentiate between genetic and epigenetic components in respect to religion, attributed to the so-called "God-gene" [16] is to see how, against the background of DNA, the experience of life

in facing the unknown leads to the acceptance of laws of science at the level at which God is invoked for the unexplained. Between the headlines that attracted numerous readers and the proper subject of genetic investigation, there is a huge difference.

To know is the most striking evidence for the effort of individuals to go beyond knowing who they are. This means to see, to listen, to smell, to touch, to taste—if we limit ourselves to the discrete description to the senses. Stepping out of oneself means to experience the immediate environment of existence. Reproduction preserves life, but in order for it to take place, two distinct entities need to know each other. Sexuality is the expression of this. Anticipation drives the process: there is purpose, and there are possible ways to achieve it.

Which genes, and which environmental cues are at work in the process is a matter that molecular biologists pursue. Brain research focused on what explains attraction. Empirical evidence from the study of sexuality (in its many forms) suggests that reward is part of the process. Knowing is knowing for some purpose. Anticipatory processes engage the organism's entirety. Therefore, data regarding anticipatory performance is pertinent to understanding how the organism acts as one entity. Genetics can explain aspects of the holistic perspective. The much-commented book, *The God Gene*, by the molecular biologist Dean Hamer, of the National Institutes of Health, reveals that a particular gene (SLC18A2) might explain spirituality. The inference is simple: the gene (also labeled VMAT2) partakes in the movement of monoamines. And voilà the magic: it is also involved in monoamine modulation through which psychiatric drugs might trigger all kinds of experiences. Genetics meets brain research!

Let us simplify. It is possible that plants containing such substances were discovered by some organism. It is how to know becomes reality: try, try again. That experiences similar to ingesting psilocybin might be of the same nature a ritualistic or religious events is at best conjecture. Not subject to question is the striving for, going beyond the immediate, searching. And this is anticipation at work. But what makes posing genetics-focused questions relevant is the realization that, in the end, the interplay between being and being in the world explains the necessity of the act of knowing. Therefore, not a gene, not a DNA, not a genome, and not the brain alone, but the interplay with the world explains the never-ending quest to ask more questions. Some questions are addressed to the unknown; some question the known; some establish new knowledge domains. Religion is one of them. Once upon a time, alchemy was one of them. Preformation and phlogiston theory are yet another example. Or vitalism. Transcending one's own borderline is to place oneself is the environment. Epigenetics originates from here.

Before genetic reductionism kicked in, God was "revealed": in images supposed to represent how we think. In association with measurement technology focused on neural activity, many hypotheses (including the famous brain mirrors [17]) were advanced. Religion became a matter of neurons firing on neurons aligning (from the faithful to the proselyte). Much has been written on this because fMRI devices, like sequencing devices, are an investment in search of ways to capitalize on them. Here we shall only recap the major findings, with no intention of undermining or questioning their legitimacy. May the God-Brain movement (by no means a closed

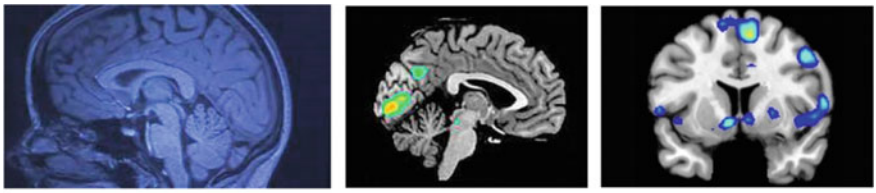

Fig. 4 How the brain registers religion. **a** Religious thought can trigger reward processes. **b** To the brain, God is just another guy (NPR). **c** This is your brain on God (Utah Health services)

chapter) serve as a warning regarding the traps of technology in search of questions (and public exposure through "hot" topics).

To start the discussion, let us consider some images.

These images are referenced to the source for a simple reason: they document various angles from which God/gods/religion became subject to fMRI representations (Fig. 4). The neuroscience of such a focus was sometimes called neuro-theology, or spiritual neuroscience. Without entering the details of the various experiments and the data generated, the following can be said:

1. The hypothalamus, amygdala, and the hippocampus were identified as the regions of the brain where divinity/religious thoughts "reside;"
2. Evidence from metabolic brain scans identified localized functional specialization of certain regions of the brain, in particular, the ventro-medial prefrontal cortex (vMPFC).
3. Once genetic focus took center stage, i.e., once a particular kind of proteins (the VMAT2) were identified, researchers of brain activity located VMAT2 proteins on synaptic vehicles. These proteins are involved in how monoamine neurotransmitters make their way to/from neurons into vesicles. This result did away with the hypothesis of a "Godspot" suggesting that multiple areas of the brain partake in the experience of religious practices.

From the perspective of the hypothesis we advanced, *the drive to know*, not a particular brain location or configuration of neurons, explains God and religion. The drive to know, connected to survival, is itself an anticipatory activity Increased awareness of the outside world and of others ("social awareness") is conducive to subduing aggression. Evidently, compassion is not an automatic outcome. It reflects the role that others play. Aggression has the opposite effect. Experience of useful (cooperative) efforts and of harmful (aggressive) interaction affect neuroplasticity. The brain learns, and in the process it changes.

The fact that religion extends from the multisensory experience of rituals explains why it remains not only a reflective practice, but also one of expression. It contains words, gestures, sound, and various images. Over time, in asking for answers from god/gods or religion, it assumes its aesthetic autonomy. It is expressed in language and in art. Prayer is a particular form of this practice of expression. Reward is part of

the larger picture, which will eventually attract the attention of those who, beyond the focus on neuroscience, got involved in the genetics and epigenetics of religion. The fact that religion, as part of the broader practice of human beings shaping their own identity, is by necessity anticipatory is the most important take-away.

4 Epigenetics

Ganesan [18], and many others trace the history and development of epigenetics back to Aristotle. In his view, Aristotle claimed that there is purpose, and thus the world seems to have a maker, which is usually the accepted view among the religious. Feynman could claim, "I told you so!" But his explanation will be overwritten as more knowledge is acquired. Although the modern science of epigenetics is relatively new [19], and still subject to questioning [20], it has attracted the attention of the scientific community. Some researchers are already looking into "reprogramming," through epigenetic changes, behaviors such as eating disorders, addiction, mental illness, memory decline, muscle biology, liver biology [21] (see also: https://ihec-epigenomes.org/). Suicide, affecting the young, as well as a great number of military service people, prompted attention because so far, the inclination to end one's life appears hidden: Is it in the genes? Others, not surprisingly, are looking at spiritual factors and their role in the life of individuals and societies. The epigenetics of solidarity—especially in extremely divisive communities, that all kinds of demographic research has tried to describe through numbers—is driven by meaning. Is it advantageous to help one another, or is it better to hate each other? The meaning of cooperation goes well beyond the numbers describing it.

On a parallel path, the role played by anticipation in surviving in various environments is the subject of in-depth research carried out by a significant number of researchers not only in the life sciences [e.g., 22–25]. Given the fact that anticipation is critical to survival, why should the question arise as to whether it is genetic or epigenetic—as long as it is a characteristic of human action? A first important distinction (along The Ethics of Terminology guiding us here): the question of whether anticipation is genetic or epigenetic [26] should not be confused with "genetic anticipation." This diagnostic refers to disorders passed on to future generations genetically (a definition is given in [27]) in sequence of shorter incidence and higher intensity. Ilkka Kronholm [28] published the findings of her investigation of this question, concluding that anticipation is epigenetic, and its functioning is dependent on environmental cues. Prior to Kronholm's report, Luo et al. [29] reported on well-being and anticipation. In short: "Anticipation for future [sic!] confers great benefits to human well-being and mental health." Their use of fMRI revealed that "… the anticipation of positive events is a key element of well-being" (p. 2), more so even than anticipation of neutral events—and even less anticipation of negative events (no matter how necessary such anticipation might be). Our lab experiments were focused on

successful performance, which is only one aspect of anticipation. Avoidance of self-harm, (i.e., no self-destructive actions) is another aspect. Neither can be traced back to genetics. Both are epigenetic in nature, i.e., triggered from outside.

Empirical evidence extending from anthropology to ethics to computer science, suggests that religiosity (God-related or any other form) affects the ways humans function in society. There is no magic formula, such as the more religious a person, the higher the performance. Although, for instance, among the religious, suicide rates are lower than that of the rest of society. The statement: "Religion A is more conducive than religion B to high performance" is rather an expression of bias. And so is "No religion at all (atheism) is more effective." After all, the history of religion itself is rather contradictory. Accomplishments connected to practicing religion (e.g., being charitable), but also to behaviors of extreme consequences (such as wars, or oppression of those not aligned with a certain religion) are well documented. In the context in which human performance, including ethical aspects, is subjected to evaluation from the perspective of the self-reproduction of life, such empirical evidence cannot be ignored. Community life, within a certain scale, was well served by "commandments" inspired by practical rules attributed to divinity. Behavioral epigenetics (where hypotheses are usually tested on surrogates, not on human beings) provides knowledge regarding factors such as taking care of offspring, respecting one's neighbors, helping the elderly, and emotional aspects of life. It is legitimate to ask to which extent genetics, as part of science, can help us explain what, at some moment in the evolution of the species, makes religion necessary or what in our times makes it more questionable. In its broad sense, spirituality affords evolutionary advantage. In our age, spirituality plays a different role that it did way back as the human condition was defined.

The pragmatics of human activity reflects the fact that "We are what we do"—which is the "school of life," i.e., the source of everything we know. Choices of means and methods corresponds to the concrete context. Anticipatory processes guide these choices: for example, "run away," "stay and fight," "help," or "hide." Genetics provided powerful tools for identifying how the change in the human being's pragmatics—from the age of foraging and hunting to what humans do now in the age of computation and synthetic biology—accrued over time in the change in genomics. Trans-generational epigenetic inheritance [30] means that environmentally, and probably socially, induced phenotypes persist over generations. Some [31] document lower baseline levels of the stress hormone cortisol in children of Holocaust survivors. Others [32] warn about overinterpretation of results. Within the controversial DNA-as-blueprint metaphor, this means that a variety of molecular processes affect gene expression and that a timeline is difficult to establish—never mind the pitfalls of surrogacy: mice and human beings. Positive and negative factors—stimulate or limit living processes. Some can lead to genetic plasticity—yet another form of adaptive behavior. Anticipation in a slowly changing environment is different in degree and in its forms of expression from that in a faster-changing context. Genetic processes can result in enhanced offspring performance (e.g., immunological defense), but also in negative influence (think about various forms of addiction).

Humankind's evolutionary path can be described in detail if the full record of anticipatory and genetic expression can be reconstructed. The focus should be on acknowledging both, and on transcending the time limits of closed-systems experiments. Once upon a time, survival took extreme forms: The ancient Greeks, for instance, abandoned the elderly no longer able to live on their own. This also reflected their views on life after death. The sense of co-dependence developed against the background of shared views or shared knowledge. Sometimes the shared knowledge is embodied in tools or in patterns of behavior. Pragmatic considerations explain the interrogations articulated over time: from perception of an enemy, of the beneficial (e.g., weather, plants, animals), or of the unknown. Neither genes nor brain configurations nor anything else subject to reductionist focus explains how sharing emerged. But through awareness of the fact that epigenetic inheritance facilitates the fitness peak of some populations [33]—expressed through successful anticipatory performance—we gain a new understanding of their condition. The evolutionary advantages of sharing over confrontation are ultimately reflected in expanding the resources available for maintaining life. Community or cohesion is not genetic, but pragmatic in nature, and as such, by necessity anticipatory. A possible future (e.g., storm, fire, drought, poisoned water) engages a community as a whole. In the process, reproduction mutates from being only the outcome of sexual drive to a process of selective attraction. Successful mating is not accidental. There is a progressive increase in what is called "intentionality." This is documented in what the history of family actually is: a record of behavioral change reflected in the genetic transcription process (with RNA involved), but not reducible to it [34]. It is in the pragmatic self-making of individuals that their encompassing profile is defined. Within this broader profile (which includes the protein profile), their genetic profile plays a particular role. The genetic clock—the record of large timescale changes in the succession of genetic bases, documents how, from reaction to interaction, the ability to question is ascertained.

5 The God of the Genome

DNA structure reflects its role in the broader dynamics of evolution. It is supposed to be chemically extremely stable, yet flexible enough to facilitate what is called gene expression. In simpler terms: there is continuity in reproduction, but also variation. From the sameness of DNA in each organism, to the uniqueness of each organism, there is a dynamic process involving al great number of parameters. Just for the sake of the argument: the situation regarding God, gods, divinity, idols, and the world in its limitless variety is similar. There are some limited ingredients (like the acids making up the DNA, each represented by a letter), and there is a generative mechanism: combine words (remember: "In the beginning was the word…." the *logos*…), combine texts, combine interpretations. A reputed scientist, Francis S. Collins—credited with discovering the genes associate with some disease, active in the Genome Project, and who worked as Director of the National Institute of

Health—ascertained: "The God of the Bible is also the God of the genome." He even asked, "…will we turn our backs on faith? […] concluding that traditional religious symbols can now be replaced by engraving the double helix on our alters?" [35]. Feynman, in describing the role of religion, explained why the more we know, the less we need God. What he did not notice was the process of turning our own constructs (in this case the DNA) into arguments in favor of religion. The theology of science–declare some entity which humans constructed to be real—leads in our days to circular thinking.

Neural correlates (e.g., [36]) of religions and spiritual experience across cultures and faith traditions and behavioral measurements document an extremely delicate process. Measuring disturbs; it also reflects, like in a mirror, those who measure and their views, no matter how much they try to remain "objective." We are exceptionally successful in measuring, but not by far as successful in transforming the data into actionable knowledge. Does gene sequencing break this vicious cycle? The organism is in a renewal dynamic with the DNA-RNA-protein one directional path postulated.

The four acids, identified through the letters G, C, A, T, (which are the initials of their names), also known as bases, are grouped in codons: groups of three. There are 64 possible ways to combine the four bases into groups of three (Fig. 5). The translation from codons to amino acids uses only 20 of them. It turns out that some genetic sequences lead to an efficient way of making proteins; others are less efficient. The environment, not the chemical make-up, explains why more protein is produced than necessary. The transfer RNA (tRNA) carries amino acids to the cell, which recognizes, in advance, i.e., in anticipation, the need to compensate for a poor environment. Anticipation is also present in avoiding the high cost (of energy and chemistry) of making proteins when not necessary.

Current sequencing means and methods embody the reactive-deterministic view inherent in the Turing machine. Therefore, the non-deterministic anticipatory component is usually omitted. Empirical evidence, i.e., documenting phenomena which take place in open systems (the living in the world), is of the nature of an open-ended record. To reduce it to genetic processes affords data describing parts of phenomena, but not their integrated nature. Let us consider, by way of an example, the energy consumed and the data collected in what can be described as adaptive performance. Indeed, in anticipation of adverse conditions, swarms of migrating birds or of fish change, respectively, flight altitude or swimming depth. Actually,

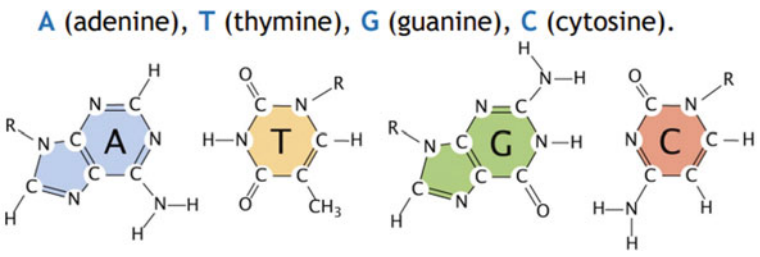

Fig. 5 From the acids of DNA to the convention of labeling them

they change the timeline (starting time)—and often the trajectory. It is quite clear that genetic processes of all kinds underlie Such adaptive behavior is related to genetic processes. Nevertheless, this behavior cannot be reduced to the genetic bases or to codons, to DNA, RNA (mRNA or tRNA), or to the process through which proteins are made in the real time of adaptive performance. There is anticipation at work informed by the integration of environmental cues of many types. Epigenetics might be the conduit assuming that it can trigger genetic processes not in reaction to stimuli but in anticipation of stimuli.

Anticipatory abilities, upon which higher performance (i.e., survival) depends, are acquired through learning. The organism is part of the world and interacts with it in terms of learning what it might mean for a particular organism or for an aggregate. The aggregate behavior (such as that of a swarm, of an ant colony, of communities, etc.) reflects the interdependence of organisms. If nothing else, the preparation for long migrations illustrates the nature of learning [37, 38]. So does the behavior of a beehive, and of those that are part of it, including its biome. And so do various forms of interaction that result in families, tribes, clans, communities, etc.

The notion of a "God gene," or of the genetics of spiritual activity is connected to human beings interacting. Francis Collins (among many reputable scientists) sees the entire universe through the "eyeglasses" of faith. Feynman, wearing the "eyeglasses" of quantum mechanics, would recognize that the complexity of life still escapes science, and thus remains a territory of explanation not aligned with scientific knowledge accumulated to date. The conclusion: We need better science. Indeed, wherever religion is invoked in matters pertaining to the dynamics of the world, the cry for a more adequate science should be heard. Religion is part of culture; as culture is changed by human activity (scientific progress included) the role of religion changes.

Within the anticipatory perspective, the spiritual—as religion or as aesthetic expression, ideas, the rich universe of feelings and emotions—is identified in acknowledging the possible future. It is against the background of culture that humans get access to understanding the very complex nature of all anticipation actions that contribute to the phenotype. If indeed the quest to know drives the human being, this awareness translates into representations that include not only atoms, electrons, chemical elements, and forces, but also meaning. Whether of religious or spiritual nature, or of other motivations (such as the competitive nature of everything that is alive), ethics—the compass of human action, in whose absence humanity dissolves—by necessity guides anticipatory actions. At the level of genetics, there is no room for it (or, as we shall see, Laplace would say: No need for this hypothesis).

6 The Last Question

It is understandable that questions pertaining to accepting or rejecting a higher authority (or a plurality of them) have been posed within a variety of scientific horizons. Laplace took note that the physics of the universe depended, in Newton's

system, on God's active role in maintaining the permanence of planetary movement. Moreover, Newton, like so many others, left the issue of the origin of the solar system open. The seductive narrative of Napoleon's asking the author of *Du Système du Monde* (The System of the World) whether it is true that God was missing in the explanation offers an indirect argument to our hypothesis. Laplace is direct: I had no need for that hypothesis. Yes, for him, everything was a nebula of extremely hot gas. This is the origin, the start. Lagrange, a no less impressive mathematician than Laplace, presumably was assuaging the emperor's worries: "Ah, it is a fine hypothesis; it explains many things." To know is by necessity to know for the future. Explaining, as Laplace did, how things work, is inconsequential. Neither the physics of Newton nor of Laplace, never mind of Einstein or of the quantum mechanics model transcend the initial question: the WHY? of the dynamics of the universe.

But there is in addition to the universe as subject of inquiry for physics and chemistry, also the universe of life—an ever-changing reality of a causality different from that of the solar system, of gravity, or of quantum phenomena. God was not a hypothesis in the ideas leading to models of organisms as machines, or as nothing other than the expression of their material composition. But unexpectedly, on examining physiology, anatomy, the brain, the chemistry of life (which is what DNA is part of), the WHY? question became a real avalanche of missing answers. Explanations are not what the divine authority is asked to provide: God, tell us why things fall down. God, tell us why people get sick. God, tell us who will become a genius or fail some exam. It is an infatuation with the future: survival, reproduction, well-being that leads to some explanations.

All activities upon which the future depends are guided by encompassing anticipatory actions. The line of argument in supporting the hypothesis articulated in this paper is straightforward: identify the variable; define the observables, present the findings (data and meaning) from descriptions of anticipatory processes; frame the God-hypothesis (or the arguments against it) in brain research and genetics. The larger questions regarding the future of religion were not part of our considerations. Only imagine, *ad absurdum*, that science (in whatever way it is understood and practiced) answered all questions pertaining to the future. It would disappear, as God and religion would. But life is an open-ended act of creations: something that never existed before continuously comes into the world, and thus change sets forth more change. As long as there is one unanswered question, God/gods/religion will stay with us humans (or whatever we might evolve into).

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Epigenetic Processes as Anticipatory Mechanisms: Insect Polyphenism as an Exemplar

Carrie Deans

Abstract Anticipation refers to the ability to use past information and possible future scenarios to inform a current action. Anticipation is not exclusive to human cognition. In fact, as a function of living organisms, it isn't limited to cognition. Data from studies on plants, microbes, and other taxa lacking complex neural structures increasingly show that simple cellular processes and biochemical pathways can also act based on anticipation. The biological functions that facilitate anticipation are organized in ways that incorporate information about the temporal association between different environmental factors. This allows responses to be initiated ahead of environmental changes and coordinated to achieve maximum physiological efficiency. Examples of anticipatory biological processes include circadian rhythms, stress priming, hormesis, and epigenetics, to be discussed at length in this article. The defining features of anticipatory functions will be presented. The unique role that epigenetics plays in mediating intra- and inter-generational anticipation will be detailed, using examples from insect polyphenisms.

Keywords Gene methylation · Histone modification · Non-coding RNA · phenotypic plasticity

1 Anticipatory Processes[1]

How can we differentiate anticipatory processes from reactive processes? What are the defining features of each type of process? All biological processes can be described by stimulus–response relationships (S-R). Every process consists of a beginning, middle, and an end, which is initiated by a particular stimulus at a given point in time and is carried out to an endpoint. Though all biological processes are

[1] There are several definitions of anticipation. The most significant are found in [1, 2].

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bound by their S-R relationships, the number of components involved, their organization, and the timing of their interactions can vary a great deal, and it is in these details that anticipatory and reactive processes can be differentiated. To this end, the causal and temporal components of these processes will be discussed, as well as their organization, at the physiological level and at the ecological level.

1.1 Causal Properties

The causal properties of a biological process describe the relevant components and the cause-and-effect relationship between them. As mentioned, all processes have an initiating factor, or stimulus, that triggers a sequence of reactions, called the response. In many cases, a response may be initiated by several different stimuli; however, at any given time, only one stimulus is implicated in triggering a response. Mitchell et al. [3] provide their own examples of these causal properties in their discussion of different S-R relationships in regulatory systems. They outline four different S-R relationships: direct regulation, stochastic switching, symmetrical anticipatory regulation, and asymmetrical anticipatory regulation. Direct regulation describes the situation where one response has only one stimulus (S1 → R1). Stochastic switching occurs when one stimulus can initiate one of two different responses and does so randomly (S1 → R1 or R2). Symmetrical anticipatory regulation occurs when two stimuli can elicit their own respective responses or that of each other's (S1 → R1 or R2, S2 → R2 or R1). Asymmetrical anticipatory regulation occurs when two stimuli elicit their own responses, but one can also elicit the response of the other (S1 → R1, S2 → R2 or R1). Although these examples are simple representations, they encompass the general types of cause-and-effect relationships commonly seen in living organisms.

1.2 Temporal Properties

The temporal properties of biological processes describe the interactions between the components through time. The two types of temporal factors that are most relevant to anticipatory processes are ecological timing and physiological timing. Because all living things are impacted by their environment, in both beneficial and detrimental ways, the timing of environmental events has strong implications for fitness. The occurrence of different events can provide useful information about the probability of future events. For instance, when two environmental factors are temporally correlated, the timing of one factor provides information about the timing of the other. Precipitation events, for example, are commonly preceded by a detectable reduction in barometric pressure, and as a result, this decrease in pressure can be used to predict rainfall. The relationship between barometric pressure and precipitation is an example of a temporal correlation that may provide useful information to organisms whose

fitness is affected by rainfall. When temporal correlations are highly stable, they can become embedded in regulatory systems and create anticipatory mechanisms. One way this can occur is through stimulus-switching, where the regulation of a response evolves to be initiated by a new stimulus that is correlated to, but precedes, the original stimulus, thus forming the asymmetrical S-R relationship discussed above. Tagkopoulos et al. [4] provide an excellent discussion of stimulus-switching, using examples from *E. coli*, where the transcription of anaerobic respiration genes is triggered in response to temperature changes that are correlated with impending anoxic environments. They also offer in silico and experimental data to show that these correlations can produce regulatory networks that are anticipatory via natural selection.

1.3 Effects on Fitness

At the ecological level, temporal correlations between environmental factors provide living organisms the opportunity to anticipate changing possibilities in their surroundings (this also applies to cellular environments). However, for these changes to be adaptive, they must impact organismal physiology in beneficial ways. As such, the potential impacts that anticipatory processes have on physiological function must be discussed. At the physiological level, the time it takes for an organism to respond to an internal or external change, i.e., homeostatic perturbation, has important implications for that organism's fitness [5]. The evolutionary basis of anticipatory processes lies in their ability to minimize homeostatic error by improving response. Deans [6] demonstrates this using a hypothetical example that shows how the timing of different response phases operates in putative anticipatory versus reactive processes. Every biological process consists of an initial preparatory phase, during which the components required to carry out the response are produced, assembled, and/or transported, and this takes a certain amount of time (measured in milliseconds). Reactive processes are typically triggered once homeostatic error is detected, meaning that there is a direct temporal relationship between the demonstrated need for the response and the initiation of that response. For instance, cellular repair processes are initiated upon the detection of cellular damage. Under these circumstances, however, a certain amount of homeostatic error must occur before the response can be triggered and it will continue to accrue over the preparatory phase. Anticipatory processes, on the other hand, can initiate a response before homeostatic error occurs, thus reducing biological damage and increasing the efficiency of the response. This can be done if a process is regulated by a stimulus that also anticipates future homeostatic error. This allows the preparatory phase to be initiated before error occurs, which does not shorten total response time but rather coordinates the timing of these phases to limit error accumulation. A good example of this is the heat shock response in *E. coli*, where the production of heat shock proteins is not triggered in response to the presence of misfolded proteins but rather by changes in the temperature-dependent secondary structure the σ^{32} transcription factor [7]. Here, σ^{32} mRNA is used as a

temperature sensor that anticipates a probabilistic need for protein chaperones and initiates their production before they are required, thus limiting protein damage.

It is important to note that not all homeostatic error is associated with damage or negative effects on fitness. Any physiological inefficiency can be seen as a type of homeostatic error, such as those that limit growth or reproduction. As such, anticipation can also reduce homeostatic error through other processes, like those related to resource action. In line with our earlier example, if an organism is dependent on precipitation for growth and reproduction, as is the case with aquatic invertebrates that inhabit ephemeral pools, individuals that initiate growth processes based on changes in barometric pressure will be able to grow more quickly than those that wait for rainfall. This is because they will minimize the time lag associated with the preparatory phase of the response, which means they will be able to utilize the resource more efficiently. The physiological advantages of anticipatory biological processes are discussed in greater detail in [6] and [8].

Figure 1 provides a summary of the relevant characteristics of anticipatory processes discussed so far and shows how they interact to produce anticipatory action. Figure 1a shows an example of asymmetrical anticipatory regulation, where S_1 induces R_1 but S_2 is capable of initiating R_1 and R_2. These components, in and of themselves, do not constitute an anticipatory function, as the temporal relationship between stimuli are not yet defined. Figure 1b, however, shows the environmental factors that S_1 and S_2 are associated with and their relative timing in a hypothetical ecosystem. Here we can see that environmental factors 1 and 2 are temporally correlated, with factor 2 always preceding factor 1. This provides important information about the potential timing of each response under these particular environmental conditions, given their relationship to S_1 and S_2. Figure 1c shows the time course of R_1, including the relationship between response stimulation, the timing and duration of the preparatory phase, and the generation of homeostatic error. In this example, homeostatic error accumulates during the preparatory phase because stimulation coincides with the presence of the environmental factors it must respond to. Given these properties, Fig. 1d indicates two scenarios: one where R_1 is initiated by a reactive mechanism (top) and another where it is initiated in an anticipatory fashion (bottom). When R_1 is stimulated by environmental factor 1, homeostatic error accrues during the preparatory phase, but when R_1 is stimulated by environmental factor 2, which precedes environmental factor 1, the preparatory phase occurs before factor 1 is present. This results in the minimization of homeostatic error due to the coordination of the response. This hypothetical example shows how the causal and temporal components of biological processes can interact under the right ecological circumstances to produce anticipatory functions, and the potential physiological benefits of anticipatory versus reactive processes. When we consider that simple organizational changes are required to turn reactive processes into anticipatory ones, it is clear that these mechanisms need not be complex. This suggests that anticipatory processes are likely more common and play a more significant role in biological function than is currently recognized. In fact, one could argue that all biological processes could be categorized as having reactive and anticipatory functions, establishing an even more foundational role for anticipation in biology.

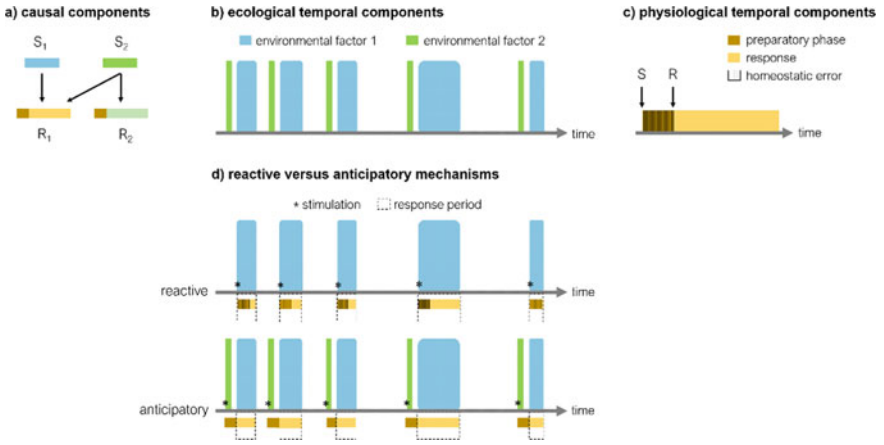

Fig. 1 An example showing the key features of anticipatory mechanisms using a hypothetical biological process that exhibits asymmetrical anticipatory regulation (**a**), where response 1 (R_1) can be stimulated by stimuli 1 (S_1) or stimuli 2 (S_2). Stimuli 1 (S_1) is associated with environmental factor 1 and stimuli 2 (S_2) with environmental factor 2, both are which are temporally-correlated with each other (**b**). Panel **c** shows the time course of response 1 (R_1), which includes a preparatory phase that must occur before the response can begin. Panel **d** shows the time course of response 1 when it is regulated in a reactive manner by environmental factor 1 and when it is regulated in an anticipatory manner by environmental factor 2. Environmental factor 2 initiates response 1 earlier than environmental factor 1, such that the preparatory phase occurs before environmental factor 1 is present, resulting in reduced homeostatic error

1.4 Anticipation Across Biological Scales

Anticipation can be manifested in different ways and at different scales. It can shape art, culture, and social progress, but especially life itself [1]. Anticipation operates at different levels of biological organization, as well, from the molecular to evolutionary. At the molecular scale, we have already discussed the role that mRNA secondary structure can play in stimulating the anticipatory heat shock response in *E. coli*. Circadian rhythms, which are highly conserved across single- and multicellular organisms, allow diel cycles to be anticipated so that relevant processes can be coordinated at the organismal level. Natural selection itself can be viewed as an anticipatory process in that it preserves the genes that are expected to be useful in the next generation, based on their past and current utility [9]. Although natural selection has strong impacts on biological form and function, it is not a biological process but rather an emergent property of biological interactions. The anticipatory processes discussed so far relate to those encoded in an organism's DNA. These genetic mechanisms, though effective, are largely intractable, as an organism's DNA remains unchanged throughout its life, with the exception of random mutations. This means that the anticipatory capabilities derived from genetic sources are effectively set at birth and can only operate intra-generationally. This isn't the only way that anticipation can occur, however. Epigenetic regulation provides an additional layer

of flexibility and, as a result, can serve as a unique kind of anticipatory function that is even more responsive. Before we get into the details about the potential anticipatory function of epigenetic processes, we must first thoroughly clarify what epigenetics is.

2 Definition of Epigenetics

Despite its ever-increasing popularity, the field of epigenetics is riddled with semantic issues. Since its inception in the early 1940s [10], the term *epigenetics* has taken on a different definition in virtually every scientific field. The ecological disciplines optfor broader definitions relating to gene regulation and gene-by-environment interactions; developmental biologists focus on canalization and cellular programming; and geneticists concentrate on heritable expression states. A thorough discussion of these different definitions and their criteria is beyond the scope of this paper, but see [11–14]. Agreement across definitions can be found in the acknowledgment that epigenetic processes impact gene expression in ways that are not dependent on DNA sequence, typically through changes in the accessibility of transcriptional machinery to DNA.

Perhaps the greatest point of contention across definitions centers on whether epigenetic marks or expression states must be heritable, and this occurs because epigenetic marks can influence gene expression both intra- and inter-generationally [13, 15, 16]. Thus, epigenetics can be seen as either a mediator of gene regulation or something more. Gene methylation, histone modifications, and non-coding RNAs can affect gene expression at the cellular and/or organismal level, but some marks can also change over the course of an organism's life and be passed on to the next generation in some cases. So, what is the primary role of these epigenetic marks? Are they simply another aspect of gene regulation or do they represent a novel type of inheritance, a kind of inter-generational memory? These questions and many others remain unanswered, largely because we still don't fully understand the causal and/or temporal aspects of epigenetic processes. For example, we don't know whether epigenetic marks are a cause or consequence of gene expression [13, 17]. What determines which marks are maintained? What determines whether new marks are made or old one destroyed? Are they impacted by environmental factors, and if so, can they persist across mitosis and/or meiosis? To most of these questions, the answer appears to be: It depends. It depends on the organism, the gene region, the type of epigenetic mark, and in many cases, seemingly on randomness. Despite these many limitations, one thing is clear: Whether they operate on an intra- and inter-generational basis, epigenetic processes have the capacity to serve as anticipatory biological mechanisms.

3 Epigenetic Regulation as an Anticipatory Mechanism

Epigenetics plays an important role in regulating tissue-specific and global gene expression through pre- and post-transcriptional mechanisms. Gene methylation and histone modifications affect the accessibility of DNA to transcriptional machinery, thereby enhancing or suppressing expression, while small RNAs primarily regulate expression post-transcriptionally through influencing mRNA translation and/or stability [18–21]. When it comes to heritability, DNA methylation and histone modifications can be maintained across mitosis, meiosis, and fertilization. The ability of small RNAs to be transferred throughout these processes is unknown, but it is more likely that they are regulated by inherited methylation and histone modifications in daughter cells or offspring than are physically transferred. Although some studies have documented a relationship between specific pre-/post-natal environments and persistent changes in DNA methylation and/or histone modifications, the underlying mechanisms connecting environmental factors to epigenetic alterations have yet to be elucidated. In any case, there are numerous examples of environmentally induced responses being, at least partially, mediated by epigenetic processes. It is perhaps most likely that environmentally induced changes in gene regulation may alter epigenetic marks through some process, thus serving as the connection between environmental conditions and epigenetic changes. At this point, however, the relationship between environmental change and epigenetic change is ambiguous.

Epigenetic processes have the capacity to act as anticipatory processes because they possess the causal and temporal characteristics required for anticipation discussed above. Figure 2 outlines the relationship between these causal and temporal factors in epigenetic processes. As strong regulators of gene expression, epigenetic processes can affect virtually all aspects of organismal function, significantly impacting fitness. Epigenetic processes do differ in important ways, however, from the genetically mediated anticipatory processes we have discussed so far. For one, epigenetic marks are much more dynamic than DNA sequence changes and can produce expressional dynamics on a faster timescale. While processes encoded in DNA are set a birth, epigenetic marks can change over the lifetime of a cell or organism, depending on the conditions they experience [22–24]. While functional changes to DNA may take very many, often hundreds of, generations to evolve, epigenetic processes can produce expressional changes within an individual's lifetime [25–28].

Genetic and epigenetic anticipatory processes also differ in their S-R relationships. While genetically mediated processes are often directly triggered by environmental stimuli, epigenetic processes appear to be primarily mediated by internal signals. The information held and transmitted in epigenetic marks do not contain any actual information about environmental patterns, but rather information about past transcriptional responses to environmental conditions. In many ways, this is similar to circadian rhythms, which are initiated by an internal clock that is periodically

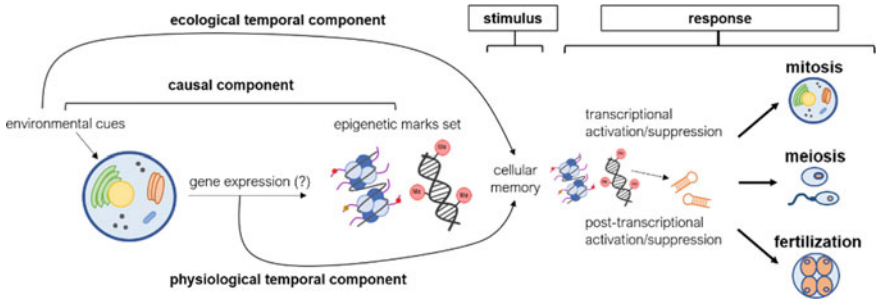

Fig. 2 A diagram showing how the key features of anticipatory mechanisms relate to epigenetic processes. Environmental cues impact gene regulation, thereby altering epigenetic marks, such as gene methylation and histone modifications. This process creates a causal relationship between environmental change and a biological response, while the epigenetic marks serve as a cellular memory of this relationship, thereby integrating the ecological and physiological temporal components. These marks then become the stimuli for regulating gene expression, the response, in new cells and/or individuals

entrained by environmental stimuli. Here, rather than constantly coordinating biological processes with external signals, circadian-regulated processes respond more efficiently to internal cues that model predictable environmental changes. Epigenetics may operate in a similar manner, using epigenetic marks as a temporal record of gene expression rather than as a template for future expressional programs. In this way, epigenetic marks serve as a kind of cellular memory that carries information about past expression, which contains information about the genes expressed and their level of expression over time. As Fig. 2 shows, these epigenetic marks effectively integrate ecological and physiological temporal components into one higher-order signal, which serves as the stimulus for epigenetically controlled responses. Because epigenetic marks are presumably set by the activity of genomic responses to external stimuli, i.e., gene regulation patterns, they implicate gene regions that have a demonstrated biological utility. This is an important characteristic because it provides a justification for expecting a future utility. In this way, epigenetic marks contain probabilistic information about the likelihood that certain genes will need to be expressed/suppressed in the future so that their accessibility can be regulated accordingly. This process is similar to that of stress priming, sometimes called sensitization, where stress-response pathways remain in a suspended state after being initiated so that they can be carried out faster upon re-initiation. In fact, histone modifications have been implicated in stress priming in plants, along with the storage of conjugated signaling molecules [6, 29–35]. Interestingly, stress priming and circadian rhythms have both been discussed as key examples of anticipatory processes [5, 6, 36].

A final difference between epigenetic and genetically based processes, is that they can operate inter-generationally. Existing epigenetic marks help regulate gene expression within a cell or organism, but in many cases, these marks persist across

mitosis, meiosis, and fertilization. While the processes that perpetuate these marks are beginning to be explained, the exact process and its regulatory factors are still unknown. During mitosis, the reestablishment of histone modifications associated with newly replicated DNA is believed to be directed by histone modifying enzymes, which may or may not remain attached to the DNA during replication. Data suggest that some marks are reestablished right after DNA replication and more faithfully maintained, while others are gradually reestablished throughout the cell cycle [37, 38]. Post-mitotic remethylation is carried out by DNMT1 on newly synthesized DNA and directed by loci that are hemimethylated [39, 40]. Global demethylation occurs in gametes during germ cell development and again in early embryos. In germ cells, somatic methylation patterns are erased and replaced with sex-specific patters. In embryos, gametic methylation patterns are erased and reestablished at implantation through de novo methylation performed by DNMT3 [40]. The factors that direct this remethylation are not known, but chromatin structure is believed to play a major role, involving histone modifications and other enzymes and modifiers [41, 42]. Beyond gene methylation and histone modifications, cytoplasmic and maternal/paternal compounds can be transferred to offspring or deposited in and around eggs and can also modulate expressional profiles inter-generationally.

4 Epigenetic Regulation in Insects

Although insects possess the same general epigenetic processes as vertebrates, i.e., gene methylation, histone modifications, and non-coding RNAs, there are some key differences in the range, characteristics, and functions of these markers. Some epigenetic mechanisms are not found across all insect orders, neither do they play the same role as in mammalian systems. These differences make insect models ideal for investigating some questions, but less suitable for others.

Most epigenetic research has focused on vertebrate models, particularly mammals. While cytosine methylation ranges from 5 to 30% across bird, mammalian, fish, amphibian, and plant taxa, insect genomes show much more limited methylation, at 0–3% [43, 44]. Despite having lower overall methylation rates, methylation appears to be more gene-targeted in insects than in mammals [45]. Among insects, gene methylation is more extensive in the genomes of hemimetabolous insects, such as those in the orders Blattodea, Hemiptera, and Orthoptera, but reduced in the holometabolous orders of Lepidoptera, Coleoptera, and Hymenopteran, and almost entirely absent in Collembola, Diptera, and Streptsiptera [43, 46, 47]. Rather than being associated with gene promoters, as is typical for mammals, methylation in insect genomes tends to occur within gene bodies, including both introns and exons close to transcription initiation sites [45]. This suggests that methylation may play an important role in alternative splicing. Also, the intragenic methylation observed in insects typically enhances gene expression, whereas in mammals, methylation is more strongly associated with repression, largely due to differences in methylation sites [48–51]. Some insects exhibit different methyltransferase enzymes than vertebrates. Mammals possess two types of DNA methyltransferases: DNMT1, which

maintains existing methylation patterns across mitosis/meiosis; and the DNMT3 family, which is responsible for new de novo methylation marks. Mammals also contain a DNMT2 enzyme, which is involved in tRNA methylation [52, 53]. All insects except dipterans possess a DNMT1 ortholog; however, dipterans do have a DNMT2 enzyme that also interacts with tRNAs but does not participate in DNA methylation. All insect orders outside of Blattodea, Coleoptera, Hemiptera, and Hymenoptera lack DNMT3 orthologs but still exhibit gene methylation [46, 54]. This suggests that insects either have other unknown methyltransferase enzymes that only perform maintenance methylation after cell replication, or that DNMT1 orthologs are capable of performing both de novo and maintenance functions [43, 46, 54]. However, at this time, it is not known how DNA methylation operates in these species.

Histone modifications and non-coding RNAs are more highly conserved between vertebrates and invertebrates (and plants) than gene methylation [55, 56]. Although histone modifications have not been studied as extensively in insects outside of *Drosophila*, they appear to operate similarly to other taxa, despite some variability in the presence and/or number of genes encoding specific enzymes, such as histone acetyltransferases [57, 58]. Similar histone variants are also found in both insects and mammals [45, 59, 60]. Non-coding RNAs also operate similarly in insects as in other taxa; however, insects have been particularly useful for establishing the heritability of specific types of non-coding RNAs. For instance, PIWI-interacting RNAs (piRNAs) were first discovered in *Drosophila melanogaster* in association with infertility [61] but have subsequently been shown to play an important role in controlling transposable elements (TEs) by promoting the formation of heterochromatin near TEs [62, 63]. piRNA caches are also transferred to developing embryos to facilitate the re-establishment of chromatin states in offspring [64–67]. Small-interfering RNA (siRNA) and long non-coding RNA (lncRNA) are also implicated in X chromosome inactivation, representing an inter-generational epigenetic mechanism [20, 68].

5 Insect Polyphenism: Epigenetics as Anticipatory Mechanisms

Differences in DNA sequence alone cannot account for the wide range of phenotypic diversity that exists across lifeforms [26]. Among animals, insects possess a variety of life history traits that are not typically found in other taxa. This is likely due to their intense co-evolutionary history with host plants and natural enemies. Insects display an impressive degree of phenotypic plasticity, which includes many polyphenisms, whereby genetically similar or identical individuals develop unique morphologies and/or behaviors in response to different environmental conditions. Epigenetic mechanisms play an important role in regulating this variability, and although insect models have been used to study other aspects of epigenetics [43–45], their many polyphenisms make them excellent candidates for studying the role of epigenetics in genotype–phenotype interactions and phenotypic plasticity.

Polyphenisms related to caste determination, seasonal change, and dispersal capability are well documented in insect taxa, and these involve widespread changes in both morphology and behavior. Figure 3 shows a summary of these polyphenisms and the different morphological states they can produce. In many cases, genetically identical individuals develop very different irreversible phenotypes based on epigenetic programming. The role of gene methylation, histone modifications, and non-coding RNAs in insect polyphenisms has been investigated using observational, often correlative, studies and, in fewer cases, experimentation. In this section, I will discuss some examples of insect polyphenism, explore the evidence for epigenetic involvement in these examples, and discuss how these processes can serve as anticipatory mechanisms mediating changes at the population, individual, and inter-generational level.

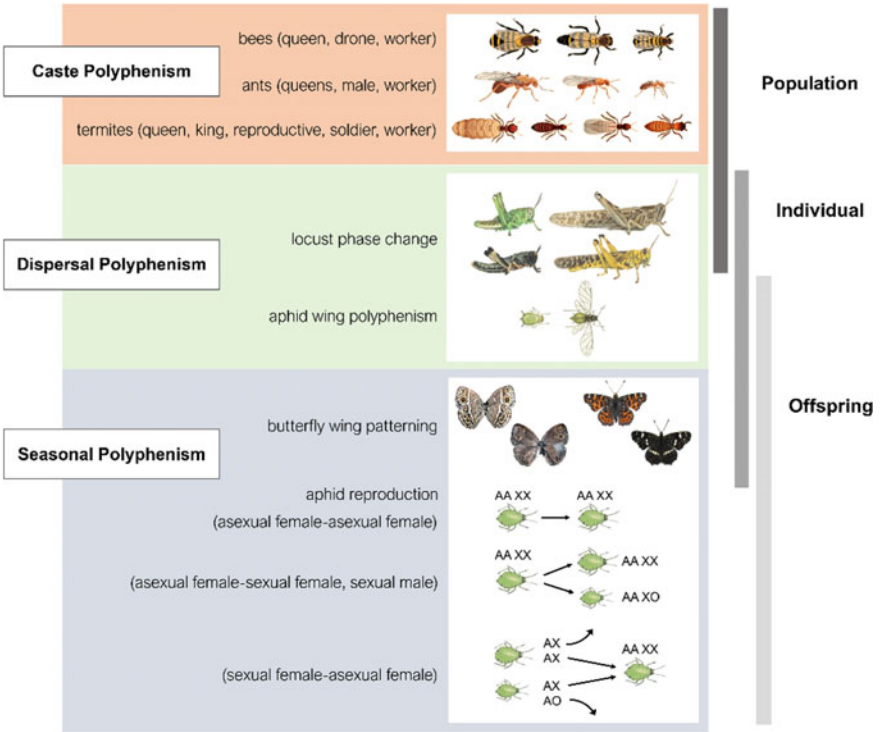

Fig. 3 A summary of insect polyphenisms, highlighting variability in castes, winged morphs, wing coloration, and reproductive systems. The vertical bars on the side indicate the scale over which specific polypheisms occur across, including changes at the population, individual, and inter-generational levels

5.1 Caste Determination

The life history of eusocial insects, which includes species of bees, wasps, ants, and termites, employs a division of labor among colony members, whereby different individuals perform different tasks and are relegated to different castes. In bees and ants, males, called drones, are derived from unfertilized haploid eggs, while females are produced from diploid embryos. Females are fated to one of two general castes that vary in many traits but are not dependent on genomic factors [69]. A female can either become a worker, which is short-lived and incapable of producing eggs, or a queen, which is long-lived and fully reproductive [70]. In termites, all castes are diploid and are divided between sterile young workers, which later become soldiers, or reproductive alates that contribute to the colony or disperse and form new colonies. The ability of workers to become secondary reproductive alates is also possible under certain conditions [71]. Figure 3 shows examples of these different castes in three eusocial insects. Caste determination typically occurs in early larval stages and is irreversible for bees and ants, while in some termite species caste development can be reversed to a certain extent [72, 73]. Caste relegation is genetically determined in some species but is more often dictated by environmental factors, such as larval nutrition, temperature, social interaction, or pheromone signaling. Although genetic variability between individuals can impact the thresholds that trigger caste divisions, epigenetic processes are largely responsible for regulating the traits involved in different caste functions [74–76]. As a result, gene methylation, histone modifications, and non-coding RNAs have all been implicated in the development and maintenance of caste differentiation.

All social insects possess the DNMT1 gene required for the maintenance of methylation marks and the DNMT3 gene needed for creating de novo methyl marks [76, 77]. Across social insect species, gene methylation appears to play a similar role in mediating gene expression associated with caste determination, although methylation differences between castes do vary across bees, ants, and termites [76]. As mentioned, gene methylation typically occurs within gene regions, is associated with increased expression, and largely regulates transcription through alternative splicing. Genes involved in caste specificity tend to show greater methylation in eusocial insects [78, 79]; however, caste-specific methylation patterns are much stronger in honeybees and termites than in ants [76]. Over 550 genes in the honeybee (*Apis mellifera*) brain showed differential methylation between worker and queen bees [80], and overall methylation was much more prominent in bee larva than adults [81]. The termite *Zootermopsis nevadensis*, which exhibits one of the highest DNA methylation rates in insects (12% of global CpG and 58% of exonic CpGs), exhibited 2700 genes that were differentially methylated across worker and reproductive alates [69, 76]. Also, almost all the genes found to be methylated in *C. floridanus* and *A. mellifera* had methylated orthologs in *Z. nevadensis*. Conversely, few differences in methylation patterns were found across castes in the ant species *Camponotus floridanus* and *Harpegnathos saltator*; however, several genes conserved in both species did show some caste-specific methylation [82]. In termites, several genes related to epigenetic

processes, such as DNMT3 and other enzymes required for histone modifications, were found to be selectively expressed in the reproductive organs of *Reticulitermes speratus* king and queens, suggesting that gene methylation and histone modifications play a role in regulating reproductive capacity in termite castes [83]. Unfortunately, most research on epigenetic control of caste determination in termites has focused on changes in the expression of genes involved in epigenetic processes, rather than actual changes in gene methylation and histone modifications. Lo and Ujvari [84] were able to detect DNA methylation in the *Coptoterme lacteus* genome but were unable to find any differences in methylation between castes.

In honeybees, differences in histone modification patterns have been found in workers and queens. Dickman et al. [85] found different lysine methylation patterns on histone H3K27 and H3K36 in ovary tissue from queens versus young larvae. Wojciechowski et al. [86] also found a strong correlation between caste-specific transcription and genome-wide modifications of histone H3K4me3, H3K27ac, and H3K36me3. They also found that the queen phenotype is set before the worker phenotype and is firmly established by 96 h of age. The differential methylation of histone variant genes in honeybee brain tissue from workers and queens also suggests that DNA methylation can impact chromatin structure by regulating the production of different histone variants [80]. Royal jelly, which is preferentially fed to queen-destined larva, can also alter histone modifications. About 5% of royal jelly is made up of the fatty acid (E)-10-hydroxy-2-decenoic acid (10HDA), which acts as a histone deacetylase inhibitor. This proposes an important role for histone acetylation in establishing or maintaining queen phenotypes and provides a *mechanism* for linking larval nutrition to caste determination [87]. In ants, histone modifications have also been linked, both correlatively and experimentally, to caste determination [76, 88]. Simola et al. [89] found that the acetylation of histone H3K27 in certain gene regions was able to differentiate major and minor female workers, as well as male castes in *C. floridanus*, by impacting the expression of genes involved in muscle development and neuronal regulation. Simola et al. [90] later showed that changing histone acetylation had strong impacts on ant scouting and foraging behavior—changes that were made permanent by injecting young ant brains with acetylation inhibitors. Few studies have been done on histone modifications in termites, but Suzuki et al. [91] showed that RNAi for three histone modifying genes in the termite *Z. nevadensis* resulted in the presolider phase being extended, implicating histone modifications in regulating solider castes.

There is considerable evidence that non-coding RNAs play a significant role in caste determination. In honeybees, changes in miRNA expression have been associated with caste and age-related changes in behavior [92–94]. Liu et al. [94] found that nurse and forager bees had significantly different miRNA expression profiles, and in particular, nine miRNAs associated with neural function were differentially expressed between the castes. Of the 97 miRNAs found in bee brain tissue by Greenberg et al. [93], five were found to be significantly downregulated in nurses compared to foragers. However, overall expressional differences between nurses and foragers were less apparent in young colonies, suggesting that external factors may regulate the expression of these miRNAs. In whole body larvae samples, Ashby et al. [95]

detected 164 miRNAs, 72 of which were differentially expressed between worker and queen larvae. Micro-RNAs have also been implicated in caste determination in the more primitive bumble bee *Bombyx terrestris*; although few caste-specific genes found in *A. mellifera* were also differentially expressed between castes in *B. terrestris*, indicating little conservation [96].

Royal jelly has also been found to contain miRNAs that may play a role in the nutritional regulation of bee castes. Shi et al. [97] found different miRNA profiles in royal jelly from *A. mellifera* and *A. cerana* and confirmed that feeding *A. mellifera* larvae both types of jelly led to the differential expression of several miRNA target genes. In a step further, Guo et al. [98] discovered that the jelly supplied to future queens and worker larvae by nurse bees differs in miRNA profiles, with worker jelly containing a much more complex miRNA profile. They also showed that experimentally altering the miRNA mixture in royal jelly can affect mRNA expression and morphology in emerging queens. Therefore, the presence of miRNAs in royal jelly, in addition to the role that 10HDA may play in histone acetylation, offers another potential explanation for how brood feeding may regulate caste determination via epigenetic mechanisms.

Micro-RNAs also appear to function in ant and termite caste differentiation. Of the 115 and 159 miRNA genes found in the *C. floridanus* and *H. saltator* genomes, several show differential expressions across castes [99, 100]. For example, the mir-64 miRNA is up-regulated in *C. floridanus* minor workers and mir-7 in major workers, while Hsal_08142 is up-regulated in *H. saltator* non-reproductive workers and Hsal_14941 in reproductive workers [99]. Eight miRNAs were found to be differentially expressed in solider and worker *R. speratus* termites, with several found to play a similar role in hymenopteran caste determination [101]. In *R. speratus,* parental imprinting is also hypothesized to impact caste determination, whereby parthenogenically produced daughters are more likely to become future reproductives [102].

Across studies, a respectable amount of data has been generated on differences in gene methylation, histone modifications, and the expression of miRNA between castes in different eusocial insect taxa. However, much more work is needed to substantiate a causal role for epigenetics in caste determination. There is a lot of variability across studies, both in the methodologies used, scale, and biological replication [75], but also in research design. In fact, a lack of experimental data is perhaps the most serious limitation to identifying the causal elements of these processes across epigenetic research. In addition, although gene methylation, histone modifications, and miRNAs have been discussed as separate epigenetic mechanisms, resultant phenotypes undoubtedly result from the interaction of all these mechanisms, further complicating matters. For instance, there is evidence that miRNA targets are under-represented among methylated genes [95]. Also, Glastad et al. [69] found that DNA methylation and specific histone modifications were strongly correlated in *C. floridanus* and that gene expression could be more accurately predicted when both were considered together.

Despite these challenges, the regulation of different castes in eusocial insects represents a fascinating example of anticipation at the population level. All colony

members are ultimately dependent, both physiologically and genetically, on the health of the colony. Thus the anticipatory act of the queen and other workers of producing the required castes has strong impacts on all members' fitness. Nutritional and social cues are the primary determinators of caste production and taken together, they operate as external and internal sensors that modulate the size and composition of the colony to match needs and constraints. The availability of high-quality resources, such as royal jelly for bees or high-protein resources (insects and seeds) for ants, can limit the production of reproductive individuals and large workers, ensuring that the colony does not outgrow its resources. Conversely, when resources are abundant, colonies can produce more of these castes and grow the colony. Insects can also use hormones to further alter the composition of different castes in order to deal with other challenges, although the factors that impact hormonal control is not well understood. The epigenetic processes that mediate caste polyphenism offer eusocial insects the flexibility to adjust to environmental changes and to anticipate needs in ways that maximize the efficiency of the colony as a whole.

5.2 Seasonal Polyphenism

Seasonal change impose important constraints on insect life history, which can affect insect morphology, physiology, and behavior. Environmental factors, such as temperature and photoperiod, can not only have direct effects on insects, but they can also signal ensuing changes in other environmental factors, such as host or habitat loss, mate availability, habitat suitability, etc. These signals can then trigger phenotypic changes related to crypsis, diapause, reproductive capacity, and dispersal. While less information is available on the role of epigenetics in seasonal polyphenism, particularly compared to caste determination, new information is increasingly providing insights into genotype–phenotype interactions and the epigenetic regulation of inducible traits.

Two key examples of seasonal polyphenism in insects are wing patterning in butterflies and reproductive plasticity in aphids. Many different butterfly species exhibit broad phenotypic plasticity in their wing coloration and patterns and, in some of these cases, seasonal changes drive these variations. For example, the southern African butterfly, *Bicyclus anynana*, develops large eyespots on their hind wings as adults if they experience warm temperatures (~ 27 °C) in the final larval instar. Conversely, adults lack contrasting eyespots on their hind wings when larval instars experience cooler temperatures (~ 20 °C) (Fig. 3) [103, 104]. These temperatures correlate strongly to the wet and dry season are regulated by an early versus late ecdysteroid peak during larval development [76, 105]. Unfortunately, the regulation of wing patterns in this species remains unknown, with no known studies on potential epigenetic regulation. Epigenetic *mechanisms* have, however, been identified in another species, the European map butterfly (*Araschnia levana*), that displays a similar seasonal polyphenism. *A. levana* adults become either a black-and-white summer form or a black-and-orange spring form depending on larval conditions

[106]. The spring form results from larvae that pupate late in the summer and overwinter, emerging as black and orange adults in the spring. The summer form develops throughout the summer months and do not undergo diapause. Shifts in seasonal temperature and photoperiod are thought to be indicative of host plant quality, as both morphs show reduced resource uptake in the late summer, yet spring morph body composition does not differ from summer morphs, despite the physiological cost of diapause [107].

Morph determination in *A. levana* is hormonally controlled by ecdysteroid signaling throughout larval/pupal development, as with *B. anynana* [108]. However, morph-specific miRNA expression suggests that the spring morph represents a default expressional program that is altered by the repressive actions of specific miRNAs [106, 107]. In particular, a miRNA that suppresses diapause processes in the flesh fly (*Sarcophaga bullata*) was also found to be up-regulated in spring morph larvae compared to summer morphs [109, 110]. Another gene associated with diapause timing in *Bombyx mori*, TIME-EA4, was found to be up-regulated in spring morphs and partially regulated by another miRNA [110, 111]. To date, the potential role of DNA methylation or histone modifications in the regulation of these traits remains unknown.

Seasonal polyphenisms are a good example of anticipatory processes that operate at the individual level to prepare insects at early stages of development for future environments (Fig. 3). Seasonal changes in butterfly wing patterning are perhaps the most similar example to our earlier discussion of anticipatory processes, as it shows a clear connection between temporally correlated environmental cues and how they can trigger biological processes in anticipation. In the case of *B. anynana*, the temperature experienced by developing larvae indicate the type of environment that adult butterflies will face. *B. anynana* is more active during the wet season and spring morphs display bright eyespots on their dorsal wings when at rest. Summer morphs, which occur during the dry season, are much less active and display more cryptic coloration. These phenotypes are hypothesized to help adults avoid predation under different seasonal activity patterns: through crypsis when it is hot, dry, and adults are less active; and through warning coloration when it is cool, wet, and adults are more active [112, 113]. In this scenario, larval temperature is used as an indicator of future predation risk. The situation with *A. levana* appears to be a little more complex. Although differences in wing coloration are also evident across spring and summer morphs, the ecological significance of this coloration is not known. It may be related to heat absorption, predator defense, or melanization and immunity [106]. The triggering of diapause, however, is likely the more functionally significant trait regulated in this polyphenism. Here, photoperiod serves as an indicator of future temperature regimes, which dictate whether developing larvae will be able to successfully eclose or if they need to enter diapause. Regardless of whether a relationship between the physiological processes involved in diapause and wing coloration exists, the genetic and epigenetic functions that mediate overwintering plasticity represent examples of anticipation.

The switching of reproductive modes in aphids is another example of seasonal polyphenism in insects. During the spring and summer most aphid species reproduce by viviparous parthenogenesis, where females give birth to live clones of themselves. In the fall, female aphids begin to produce sexual male and oviparous female offspring, via parthenogenesis, which reproduce sexually and lay cold-hardy eggs that overwinter. These eggs then produce viviparous females in the spring [76]. The switch from producing asexual females to sexual morphs is initiated by changes in photoperiod and involves altered neuro-endocrine signaling, in particular, juvenile hormone [76, 114, 115]. Although histone modifications and gene methylation have not been causally tied to the production of asexual versus sexual offspring, observational data suggest they may play a role. Chromatin remodeling has been implicated in the production of sexual versus asexual offspring in aphids, as several genes encoding histone proteins, a histone methylase, and histone-binding protein were found to be differentially expressed in sexual and asexual embryos [116]. Sexual female aphids contain two X chromosomes (XX), while males possess only one X chromosome (XO). Increased transcriptional accessibility of X-linked genes in male aphids has also been linked to chromatin structure as a mechanism for dosage compensation [117]. Global DNA methylation patterns were shown to differ substantially between parthenogenic female and sexual male aphids, with male autosomes being hypomethylated and sex chromosomes being hypermethylated compared to females [118]. The coding region of several juvenile hormone associated genes has also been found to be methylated in pea aphid, further indicating a likely role for DNA methylation in reproductive polyphenism [119]. The connection between the production of reproductive morphs and non-coding RNAs in aphids is a little better established but still in its infancy [120]. Legeai et al. [121] documented 17 miRNAs that varied significantly between three types of female aphids: parthenogenic females producing clones, parthenogenic females producing sexual males/females, and sexual females. Four miRNAs were up-regulated in sexual-producing females compared to sexual females, while three miRNAs were down-regulated. Four miRNAs were up-regulated and one down-regulated in sexual-producing females compared to parthenogenic females. But the greatest differences was between sexual females and parthenogenic females, each of which had four miRNAs up- and down-regulated. Some of these miRNAs have associations to apoptosis and metamorphosis, but the function of most of these miRNAs remain unknown.

The ability of aphids to modulate the reproductive system of their offspring represents a unique type of anticipatory process because it involves epigenetic changes at both the individual and inter-generational scale (Fig. 3). The role that epigenetic processes play at the individual level, i.e., the ability of parthenogenic females to produce sexual progeny, is still very ambiguous. In fact, even the genetic processes involved in this switch aren't well known, but it is clear that the switch occurs at the maternal level as females experience changes in photoperiod/temperature. Aphids likely switch to sexual reproduction in the fall in order to increase the genetic diversity of their offspring before the stress of winter [115], thereby increasing the probability that some will survive. Other aphid species also produce sexual offspring in

response to stressful conditions, such as host plant changes [115, 122, 123]. Interestingly, this strategy opts for the generation of random genetic change rather than the targeted up-regulation of specific traits, which is a more common anticipatory process. This method relies on a randomized response in much the same way that stochastic switching operates in bacteria [3, 124, 125]. In any case, reproductive switching in aphids represents an anticipatory fuction that relies on the temporal relationship between shorter photoperiods and the coming of winter in order to better prepare offspring—actually the offspring of offspring—for future conditions.

5.3 Dispersal Polyphenism

The ability to escape unfavorable environmental conditions, find new resources, and mates has significant impacts on animal fitness. Insects were the first animals to evolve flight capabilities, which allowed them to occupy new habitats and expand their host usage. This capability does, however, require considerable investment in specialized structures and energy-intensive functions [126, 127]. For this reason, considerable plasticity exists in dispersal capabilities across insect taxa. In some cases, this plasticity is manifested in the development of winged and wingless morphs, and in other cases it involves changes in behavior. Two of the most well-established examples of dispersal polyphenism is phase change in locusts and wing development in aphids. In both cases, environmental stimuli trigger complex transcriptional programs that involve morphological, physiological, and behavioral changes associated, not only with dispersal, but a range of other traits, such as reproduction, immunity, longevity, learning, and sensory processes. Although the mechanisms behind many of these changes are not well-understood, epigenetic processes likely play an important role.

Perhaps the best example of dispersal polyphenism is that of phase change in locusts. Locusts, which are essentially swarming-capable grasshopper species, transition between a solitarious or gregarious morph during development in response density-dependent factors. This occurs in several locust species but has been studied most extensively in the migratory locust, *Locusta migratoria,* and the desert locust, *Schistocerca gregaria.* Gregarious morphs take part in swarming behavior and can form migrating populations that span hundreds of square kilometers [128]. The transition between solitarious and gregarious forms involve changes in morphological traits, including body size, coloration, and the shape of the eyes, wings, and hindlegs, physiological changes in metabolism, lifespan, and immunity, and neurological changes in sensory perception, learning, and aggregation behavior [129, 130]. Solitarious forms display more cryptic coloration, while gregarious forms are brightly colored. Gregarious morphs also feed on a wider host range, are more active, and fly during the day rather than at night. Phase transition occurs in response to visual and olfactory signals from conspecifics or tactile stimulation of the hindleg, which typically occurs in crowded conditions. Behavior changes related to the transition from solitarious to gregarious forms can occur within hours of stimulation, but other morphological and physiological changes take longer, sometime generations [76].

The transition from a gregarious to a solitary morph takes longer and is more dependent ancestry [131]. Although morphological changes are set at adulthood, coloration of nymphs can be reset after the adult molt to reflect current density conditions, can change within a short period after adulthood but before sexual maturity, and can be passed onto offspring [132].

Epigenetic mechanisms likely play a strong role in mediating individual changes throughout development and in mediating inter-generational effects. Gene methylation, histone modifications, and the transfer of maternal compounds to eggs have been implicated in phase transition [132]. However, as with many other examples, experimental evidence for epigenetic involvement is rarer compared to observations studies documenting changes in epigenetic marks across phenotypes. Both *S. gregaria* and *L. migratoria* possess the enzymatic components for DNA methylation and histone modification, including DNMTs, histone acetylase/deacetylase, methyltransferase, and demethylase gene homologues [133–137]. Methylation rates are higher for locusts than most other insects, with cytosine methylation rates reported at 1.3–1.4% [135]. Methylation is also associated with repetitive elements in locusts [134, 135], which is unusual in insects, as methylation typically signals increased expression rather than repression.

Two studies have measured differential DNA methylation across solitarious and gregarious locusts. Robinson et al. [138] found significant expressional differences in genes related to DNA methylation in the embryos of solitarious versus gregarious mothers. They also found that several genes associated metabolism were differentially methylated in these embryos. Wang et al. [136] reported differences in methylation in the brain tissue of 4th instar morphs, occurring in the introns of 90 genes, most of which are believed to be involved in synaptic plasticity. They also found evidence for strong historical germline methylation based on the ratio of observed to expected CpG levels.

Although the role of histone modifications in phase change has been less-studied, evidence suggests that locusts possess a broader range of histone-modifying enzymes than other insect species. Guo et al. [139] found that genes encoding histone-modifying enzymes were more differentially expressed in locust eggs and adults than in nymphal stages, and in reproductive organs, particularly the testis. In brain tissue, nine histone deacetylases and ten methyltransferases were differentially expressed between solitarious and gregarious nymphs, with most being up-regulated in solitarious morphs. Other studies in *S. gregaria* suggest that histone H3 phosphorylation, as well as the methylation and acetylation of lysine 9 and 27, may play a role in swarming behavior. The phosphorylation of histone H3 is also more common in gregarious morphs [129].

Of the 830 miRNAs documented in *L. migratoria*, about 185 have been hypothesized to be involved in phase change [140, 141]. Some miRNAs have been associated with different aspects of phase change, such as dopamine production (miRNA-133) and hatching synchronicity (miRNA-276) in gregarious morphs [142, 143], but the role of most miRNAs remains unknown. Wei et al. [140] found that gregarious morphs expressed twice the number of miRNA transcripts than solitarious forms, which expressed more endo-siRNAs and piRNA-like small RNAs. Gregarious forms also

exhibited greater expression of miRNAs shorter than 22 nucleotides, while solitarious morphs had more miRNA longer than 22 nucleotides.

Another type of epigenetic regulation that we have not yet discussed are parental effects. Cytoplasmic compounds or other molecules surrounding eggs can modulate gene expression in developing embryos and neonates. In *S. gregaria*, an unidentified pheromonal substance produced in the accessory glands of the female and deposited in the foam surrounding the eggs, or potentially inside the egg itself, has been associated gregarious morph development [132, 144]. Data from Chen et al. [145] also show that other parental effects impact egg weight. Crossing gregarious males or females that were kept in isolation with gregarious morphs of the opposite sex produced lighter eggs and nymphs with smaller femur-to-headwidth ratios. Conversely, crossing solitarious males or females kept in crowded conditions with solitarious morphs of the opposite sex produced heavier eggs and nymphs with larger femur-to-head-width ratios. This shows that both paternal and maternal conditions interact to affect traits in their offspring. It is unknown whether these inter-generational effects are due to imprinting or other epigenetic mechanisms.

Locust phase change is a unique type of anticipatory process because it operates on the individual and inter-generational level, like reproductive polyphenism in aphids, but it also has population-level implications through its impact on swarming behavior (Fig. 3). For locusts, the sight and/or tactile stimulation of other locusts signals important environmental changes. It indicates that locust density is increasing, which has important implications for the mating opportunities, the spread of disease and resource depletion. Gregariousness eventually causes locusts to swarm and migrate into new habitats, which they do effectively because swarming behavior decreases predation risk. However, as resources become scarce, swarming individuals become susceptible to cannibalism from conspecifics. In fact, data show that the eventual driver of swarm movements is due avoidance behavior associated with cannibalism [146]. In this way, the environment experienced during nymphal instars leads to the anticipatory development of several traits needed to deal with an impending crowded environment, including aposematic coloration, up-regulated immunity genes, and higher fecundity. Additionally, a crowded adult environment can produce gregarious offspring to further enhanced the fitness of the parent and its progeny.

Aphid wing polyphenism is another example of phenotypic plasticity related to dispersal (Fig. 3). Here, certain environmental conditions can induce the development of wings in immature aphids [115, 147–151], or the production of winged offspring [152]. The presence of wings in male aphids are genetically determined, while for females it depends largely on environmental factors [153]. Many different environmental stimuli can trigger wing development, including crowding (tactile stimulation) [147, 151], the presence of natural enemies (alarm pheromones and tactile stimulation of conspecific ants) [154–159], pathogens [159], temperature, and changes in photoperiod [160–163]. Wing polyphenism is also closely tied to reproduction in aphids, as winged forms are often associated with sexual morphs. Whether environmentally induced or maternally induced, winged morphs develop wings and functional flight muscles. They also exhibit longer antennae, more-developed eyes, and more elaborate sensory systems compared to wingless forms [115, 164]. They

also undergo a longer developmental and reproductive period, live longer, but exhibit lower fecundity [115, 165].

Given that wing polyphenism occurs among genetically similar individuals, identical clones in most cases, epigenetic mechanisms likely regulate these expressional programs. Despite this, little data are available on the role of epigenetic mechanisms in aphid wing polyphenism. Vellichirammal et al. [166] did find transcriptional evidence of chromatin remodeling in the heads of female aphids exposed to crowded and uncrowded conditions. However, a direct link between these potential chromatin modifications and the production of different wing morphs has not been made. An RNAi study that knocked down the expression of an ecdysone receptor in female aphids significantly affected the production of winged versus wingless progeny, substantiating a role for this hormone in mediated winged phenotypes in aphid offspring [166]. Another study also found distinct differences in alternative splicing between winged and wingless female adults and embryos [167]. Despite this, no studies so far have looked at the potential role of DNA methylation in mediating these expressional changes [76], even though a gene methylation system is present in aphids [120]. Regarding non-coding RNAs, Li et al. [168] did find that 28 miRNAs were differentially expressed in winged versus wingless grain aphids (*Sitobion avenae*). Interestingly, some of these miRNAs were also found to be differentially expressed in different reproductive aphid morphs [121], suggesting that both reproductive and wing polyphenisms are connected. In the citrus aphid (*Aphis citricidus*), a specific miRNA, aci-miR-9b, has been implicated in mediating maternally derived winged morphs due to crowding [169], and in the bird cherry-oat aphid (*Rhopalosiphum padi*), another miRNA, miR-147, has been shown experimentally to be involved in wing development across morphs [170]. Despite these advances, much more work is needed to elucidate the role of epigenetic mechanisms in aphid wing polyphenism.

As with locusts, the development of wings provides aphids with the opportunity to respond to broad changes in environmental conditions. Having the ability to disperse gives aphids, which are immobile throughout most of the year, with the option to escape predators, find new hosts, and increase resource availability for their progeny. In this way, wing polyphenism in aphids represents a singular response that can mitigate a range of different environmental issues. This is the opposite of phase polyphenism, where only a couple stimuli produce sweeping phenotypic changes, but in any case, the development of wings presupposes a need for dispersal and gives individuals an advantage in dealing with future environmental challenges.

6 Conclusion

The examples discussed above highlight the potential for epigenetic processes to act as anticipatory mechanisms in mediating a wide array of adaptive phenotypes. These mechanisms involve unique S-R relationships that integrate the temporal association between past environmental patterns and gene expression patterns and use them

to modify epigenetic marks, which can be passed on to modulate future responses more quickly and efficiently. These epigenetic marks become an internal stimulus for expressional responses. Some stimuli correspond to one or two environmental factors, while others may integrate information from many different factors. Similarly, the responses regulated may be specific and simple or extensive and complex; all through the same mechanism. The importance of epigenetic regulation in developmental processes, such as cell differentiation, imprinting, and heterochromatin formation, emphasizes the fundamental, yet underappreciated, role that anticipation plays in biology. The role of epigenetics in mediating other genotype–phenotype interactions, such as phenotypic plasticity, is also increasing, as evidenced by its involvement in the many polyphenisms discussed above. Epigenetic regulation represents one kind of anticipatory mechanism, but several others exist. It is my hope that future research on epigenetics and these other anticipatory processes will bring about a new outlook on biological mechanisms; one that goes beyond the antiquated view that all processes must be unidirectional and values the significance of biological organization. After all, until we account for the existence of anticipatory mechanisms and incorporate them into the current paradigm, we will never be able to fully understand biological complexity at a mechanistic level.

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Epigenetics and Medicine

Epigenetic Mediated Regulation of Cancer-Testis/Germline Antigen and Its Implication in Cancer Immunotherapy: A Treasure Map for Future Anticipatory Medicine

Rashmi Gupta, Bimal Prasad Jit, and Ashok Sharma

Abstract Implication and harnessing innovative therapy in targeting the molecular underpinning of solid tumors are urgently needed. Previous seminal discoveries have elucidated epigenetically regulated cancer-testis antigen expression, showing a strong and durable response in a subset of patients. Cancer testis antigen/germline antigens are an umbrella of proteins usually confined to gametes and trophoblasts and abnormally expressed in several cancers displaying strong immunogenic potential. Substantial evidence suggests that epigenetic players such as DNA methylation play a crucial role in regulating the expression of cancer antigen (CTA) and are therapeutically beneficial in cancer. A combinatorial therapeutic approach encompassing epigenetic modulators targeting mechanistic targets can exhibit therapeutic benefit in clinical settings. This article explores the mechanistic basis of regulation and expression of CTA in response to an epigenetic modulator and its role in sparking the immunotherapeutic efficacy for anticipatory medicine.

Keywords Tumor antigen · Cancer testis antigen · Epigenetic priming · Epigenetic modulator · Histone modification · Combinatorial treatment

1 Introduction

Currently, immune checkpoint blockade by monoclonal antibody as well as adoptive T cell therapy has shown to be most effective and beneficial in the clinical setting. However, most of the patients do not respond to this treatment strategy. Search for an ideal tumor antigen has been an incessant task in the cancer immunology field for the past decades. The antigen must have significant expression and strong immunogenic potential for a potential and effective tumor antigen suitable for immunotherapy. Cancer testis antigen (CTA) is the tumor protein with a restricted pattern of expression; limited to germ cells or trophoblast tissues, whereas expressed in several cancers. Based on their specific expression, which is confined to the tumor cell

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and poor expression in normal tissues, makes them a prominent target for cancer immunotherapy. Evidence shows that CTA-rich cancers like melanoma, ovarian and lung cancer, and bladder cancers are regarded as "CT rich," whereas colorectal cancer and lymphoma/leukemia as "CT-poor" tumors indicate the type of tumor, stage of patients plays a crucial role in the expression of CT antigens [1]. Although the precise mechanism governing CTA expression in cancer is yet to be elucidated, epigenetic processes like DNA hypomethylation contribute substantially to the expression and regulation of CTA in several tumors [2]. Seminal investigations have addressed upregulation of CTA in the tumor is associated with treatment with DNMT inhibitors (DNMTi) [3]. Finding has shown that epigenetic priming of tumor in combination with adoption transfer regulates the metastatic spread in immune-competent murine breast cancer model [3]. Here, we review data on the mechanistic underpinning regulating CTA expression and their implication in cancer immunotherapy.

2 CTAs Are Expressed in Cancer

More than 250 genes encode an antigen family that establishes the Cancer testis antigen, having 70 gene families [4]. CTA is primarily expressed in testicular germ cells and placenta trophoblasts, with low expression in normal adult somatic cells. The precise and ectopic expression of CTA in different cancers is shown to elicit humoral and/or cellular immune responses in cancer patients. Owing to the solid antigenic potential and tissue-specific expression of CTA, they act as a potential target for anticancer drug discovery, targeted therapy, and biomarker discovery. Multiple lines of evidence suggest that CTA actively participated in vital cellular processes, including cellular development, differentiation of stem cells, and carcinogenesis (Fig. 1). Generally, CTA is broadly divided into two groups, i.e., CT-X antigens confined to the X chromosome and non-X CTAs situated on autosomes. 10% of genes on the X chromosomes belong to CT-X types and are expressed in proliferating reproductive cells [5]. CT-X antigens like NY-ESO-1, MAGE-A, CT7/MAGE-C1, CT10/MAGE-C2, GAGE, CT47, SAGE1, and NXF2 shown to be significantly expressed in advanced stages of spermatocytes [1]. Some CT antigens are nuclear proteins and meiosis-related proteins, exclusively expressed by spermatocytes and play a key role in development. Other CT genes are expressed in developed, post-meiotic sperm cells. This class comprises COX6B2, a testis-specific isoform of the cytochrome c oxidase subunit VIb. The tumor-specific expression of CTA was observed in melanoma. Subsequently, it was reported in colorectal cancer, colon cancer, prostate cancer, pancreatic cancer, lung cancer, breast cancer, cervical cancer, ovary cancer, etc. Intriguingly, it was observed that the drosophila CT genes like *piwi (PIWIL1)* and *nanos (NANOS1) have* human orthologue and are predominantly upregulated in human cancer. It indicates that *Drosophila* can be an attractive approach to exploring the mechanistic role of cancer germline genes and their potential role in tumorigenesis.

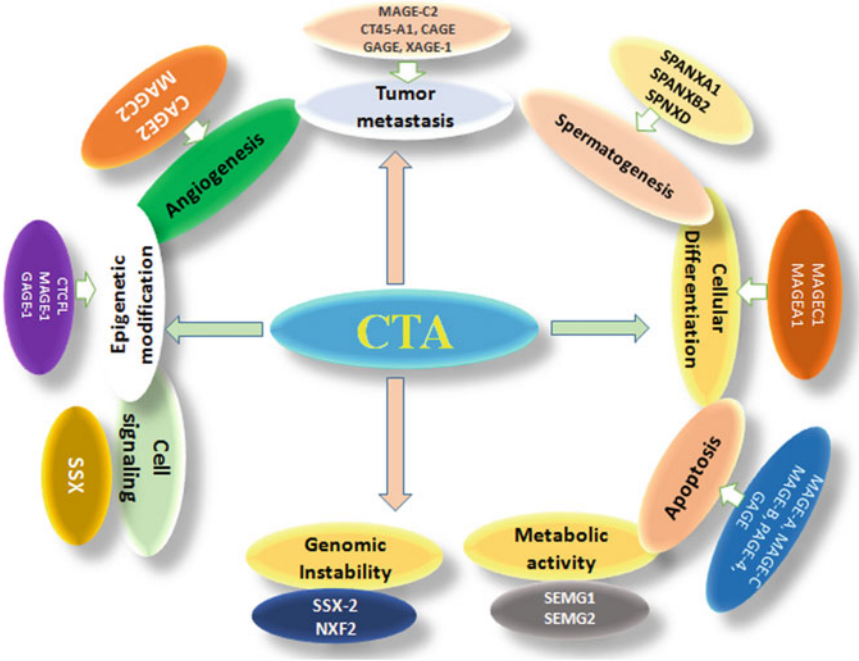

Fig. 1 Expression and potential function of different CTA in cancer

Published data suggested that the expression of some CTA is shown to associate with specific clinical phenotype. For instance, expression of the SSX CTA family is limited to benign prostate tissue, whereas the majority of these CTA (23%) are associated with the metastatic phenotype [6]. It is imperative to note that expression of CTA can be elevated in the tumors, which could not be displayed at the close of a single cell. Another scenario surface in this backdrop is the heterogenous expression of some CTA. For example, evidence from microdissection analysis of ovarian cancer specimens displays intra-tumoral heterogeneity of NY-ESO-1 [7]. This will help observe different metastatic loci originating from the main lesion due to the Intra-tumoral heterogeneity in tumors [8]. Another scenario includes a lack of specific correlation between gene and protein expression, which may contribute to differential expression of RNA and protein. However, a lack of specific antibodies can impede the detection of clinically relevant CTA. This anomalous expression of CTA can be attributed to the DNA demethylation, histone post-translational modification, and miRNA-mediated regulated events [5, 9]. Intriguingly, evidence has shown the implication of demethylating agents such as 5-aza-2-deoxycytidine in CTA expression in tumor cells [10, 11]. Previously, the role of MAGE-A1 in the induction of autologous cytotoxic T lymphocyte retort in melanoma [12]. Subsequent evidence suggested the role of several immunogenic antigens like SSX-2, with NY-ESO-1, tumor-associated antigens (TAAs), BAGE, GAGE, and MAGE family members [5, 13].

3 Epigenetics Plays a Predominant Role in CTA Expression

Epigenetic alterations play a crucial role in suppressing immune identification and immune surveillance to stimulate immune evasion via impairment in both the tumor and microenvironment [6]. Immunosuppression is a prominent feature of the global methylation pattern of the cancer genome, and it is also a common aspect that extends across heterogeneous cancer phenotypes. It involves heritable changes in the phenotype that do not involve DNA sequence alterations. Although the field encompasses three potential epigenetic modifications, DNA methylation, histone, or chromatin post-translational modifications (PTM), non-coding RNAs, DNA methylation, and histone modification are mechanisms that prominently participate in the regulation of the expression of CTA [14]. These three epigenetic modifications jointly constitute an "epigenetic code" and controls gene expression. Genetic and epigenetic factors, including the transcriptional status of various cancer cells, play a potential role in regulating CTA expression. Accumulating evidence indicates epigenetic events play a crucial role in fine-tuning the CTA expression in normal and cancer cells [15].

The CTAs are predominantly suppressed through DNA methylation in vegetal cells and undergo epigenetic activation in malignant tumors. The epigenetic processes upregulate after chemotherapy, promoting CTA-specific vaccine-mediated tumor growth and survival reduction. Moreover, these tumor-associated antigens although do not directly participate in disease development and progression [16]. The CTAs provide signals for cell division, proliferation, growth, and aggressive tumor behaviour. CTAs regulate gene expression for germ cell multiplication by altering the transcriptional and post-transcriptional machinery [17]. CTAs present specific features of neoplasia, including immune response evasion, perpetuity, DNA hypomethylation, epigenetic abnormality, invasiveness, and destruction of healthy tissues [18].

Moreover, the cancer-immune interactions increase metastatic spread and communication to new hosts. Increased expression of TAAs is one way by which epigenetic medicines restore or improve cancer cell recognition [19]. Cancer testis antigens (CTAs) are the well-studied epigenetically-regulated TAAs, and epigenetic therapy increases their expression. CTAs are expressed in embryonic and germ cells, but DNA methylation at the gene promoter silences them in mature somatic cells. Demethylation caused by DNMT can result in the re-expression of CTAs in cancer cells in various solid tumors [20]. HDACi have also been demonstrated to upregulate CTAs at a much lesser level than DNMTi. Furthermore, combined DNMTi/HDACi therapy increases CTA expression in some, but not all, cell lines; however, unlike DNMTi alone, the rise is not long-lasting [22].

Furthermore, more significant detection and destruction of malignant cells is not always the outcome of dual epigenetic therapy. The epigenetic medicines have been demonstrated to sensitize cancer cells to immune checkpoint therapy by upregulating the immunological checkpoints CTLA-4, PD-1, PD-L1, and PD-L2 on tumor cells

and TILs, potentially giving an immune escape route [23]. Furthermore, strong PD-L1 expression in tumor cells and TILs has been linked to good clinical responses to anti-PD-1/PD-L1 therapy.

4 Role of Epigenetic Modulator in the Regulation of CTA Expression

Owing to their antigenic solid potential and tumor restricted expression CTA is the potential target for immunotherapy. Multiple lines of evidence suggest that epigenetic events, especially DNA methylation and epigenetic modifications, play a prominent role in the expression and regulation of CTA in cancer. Understanding and exploring such mechanisms involved in CTA expression is of paramount importance [19].

Epigenetic agents. CTAs are regulated by epigenetic systems such as DNA methylation and histone modifications.

DNA methylation/Demethylation. DNA methylation is the most widely studied covalent epigenetic modification that plays a predominant role in regulating the expression of CTAs. In eukaryotes, DNA methylation occurs in the CpG dinucleotide of the cytosine ring preferentially situated at the 5′ promoter region of more than 50% of genes [20]. DNA methylation regulates the progression and development of several diseases by controlling lineage specification, cellular identity establishment, X chromosome inactivation, embryonic development, genomic imprinting, and epigenetic programming [21]. The methylation process is highly crucial in the ectopic depression of CTA genes. The primary mechanism includes adding a methyl group to the 5′ position of cytosine. The formation of a 5′ methylcytosine structure acts as a signal that paves the access of different transcription factors (TFs), or recruitment of methyl binding domain proteins (MBD) with histone modifications can lead to gene repression [22]. The binding of MBDs to the methylated region of the genome is accompanied by the recruitment of histone deacetylase leading to histone deacetylation in the promoter. The previous finding also elucidates the crucial role of MBD1 in the repression of the MAGE-A gene.

Deacetylation in the promoter region causes tight packing of DNA, diminishing the binding activity of transcription factors, thus inhibiting transcription. Evidence from normal somatic tissue shows an association of promoter methylation with male germ cell-specific CTA expression (MAGE-A1) [23]. The evidence initially surfaced when demethylating agent 5′-aza-2-deoxycytidine (5DC) in cultured tumor cell lines leads to induced/upregulated CTA expression. Subsequent studies' findings show that 5′-aza-2-deoxycytidine can be implemented in the upregulation of several CTAs like MAGE, GAGE, SSX2, and NY-ESO-1. [24]. The possible mechanistic role of 5-DC on CTA expression by DNA methylation has been established, and finding show 5-DC can entrap the DNMT in complex with DNA leading to progressive loss of DNA methylation leading to transcriptional blockage, thus leading to upregulation of CTAs

[25]. Furthermore, knockout of DNMT1 and DNMT3b, but neither gene alone is associated with CTA expression and promoter methylation in HCT116, colon cancer cell line. However, there is considerable disparity in the methylation status of some CTAs (for example, MAGE-A11) promoter CpG island and their expression. The epigenetic events, especially DNA methylation, illustrate CTA expression's mechanistic basis and their role in oncogenesis [26]. Earlier evidence indicates promoter DNA hypomethylation drives a key role in regulating the expression of several CTAs like NY-ESO-1, TRGA3, MAGE, and CT45 in ovarian cancer [27].

Histone Modification. Histone modification plays a critical role in the epigenetic regulation of CTA expression. Histone proteins such as H2A, H2B, H3, and H4 together form histone octamer and constitute the basic structure of nucleosomes [28]. Covalent modifications of free N terminal ends of amino acids of histone play a key role in enciphering the epigenetic landscape and thus regulating several diseases' mechanistic basis. Intriguingly, previous research has shown that the modifications of histones at a particular site could alter the transcriptional response [29]. Previous evidence suggests inhibition of HDAC and DNMT could facilitate the synergistic expression of CTA like MAGE, SSX, and NY-ESO-1 family members. HDAC could provide compactness of chromatin structure which prevents accessibility of transcription factor and RNA polymerase to DNA; thus, inhibition of HDAC is a novel target to induce CTA expression. Knockout of G9a or GLP in embryonic mouse stem cells shows elevated MAGE-A gene expression [30]. However, in human cancer cells, G9a/GLP knockdown was insufficient for CTA gene activation, indicating a cell context-dependent effect and showing that DNA methylation is a more prominent regulator of CTA expression in human cancer cells [16]. The previous study shows that potent modifications like histone H3 lysine 9 acetylation (H3K9ac), H3 serine 10 phosphorylation (H3S10ph), and H3 lysine 4 trimethylation (H3K4me3) are linked with transcriptional activation. Contrariwise, modifications like H3K27me3 and hypoacetylation of H3 and H4 are associated with transcriptional repression [31].

Furthermore, synergistic induction of NY-ESO-1 antigen expression has been reported after treatment of HDAC inhibitors such as valproic acid and 5'-aza-2'deoxycytidine DNA hypomethylation agent. Further, the results revealed that DAC and Trichostatin A treatment could induce synergistic activation of multiple BORIS isoforms, suggesting that both DNA hypomethylation and histone acetylation play a vital role in the expression of BORIS in EOC cell lines [32]. Furthermore, the Enhancer of zeste homolog (EZH), a histone methyltransferase and polycomb group protein, induces the H3K27 trimethylation mark and is associated with repression of GAGE in breast cancer cell lines. However, data are scanty regarding the prospective role of histone modifications and their crucial role in regulating CTA expression [33].

5 CTA Is the Prominent Molecular Target in Immunotherapy

Multiple lines of evidence and clinical finding have shown that immunogenicity and cancer specificity of CTA prioritized them as a prominent target in cancer immunotherapy [24, 34, 35]. The potential role of tumor-infiltration lymphocytes (TILs) is currently beneficial in clinical settings. In gastric carcinoma, TILs were positively correlated with more prolonged postoperative survival [21]. In addition to this, the role of autologous T cells in several types of malignancy exhibits profound tumoricidal activity [22]. In particular, checkpoint blockade has been clinically relevant to patients' survival [24, 25, 35]. These studies show that endogenous T cells recognize antigenic determinants- epitopes displayed by the tumor cell surface and represented by major histocompatibility complexes (MHCs). Such epitopes may be originated from viral antigens, tumor-associated antigens (TAAs), and neoantigens. Both TAA and CTA are the specific target and are exclusively used to mediate the T cell response in vitro and in vivo conditions [28, 29]. Studies have demonstrated that intrinsic T cells can recognize neoantigens expressed by cancer cells and act as a prominent target for T cell-mediated immunotherapy [26, 30, 36]. Furthermore, due to their tumor specificity and ability to bypass the central tolerance, neoantigens exhibit minimal autoimmunity toxicity, rendering them potential immunotherapy targets [37].

In addition to neoantigen, the implication of CTA in boosting efficient immunotherapy has been experienced by several clinical studies [38, 39] (Table 1). CTA-peptide conjugates were found to be vaccine candidates for cancer immunotherapy [40]. Studies have also highlighted those patients failing to respond in the first and second line of chemotherapy could benefit from CTA-dependent immunotherapy [41, 42]. Both T cells and DC are implicated and shown to be promising in CTA-based immunotherapy (Fig. 2). Encouraging results, including $CD4^+$ T cells and $CD8^+$ T cells response, were observed in diseased people for both ovarian cancer and melanoma when they were treated with the help of recombinant fowlpox-NY-ESO-1 vaccine [43, 44]. However, previous clinical trial results did not show significant results in disease-free survival (DFS) in the MAGE-A3 mediated vaccine approach in patients with lung cancer [45, 46]. It is imperative to note that co-expression of several CT proteins associated with cancer phenotype can be considered a novel strategy to target and use for efficient vaccine-induced antitumor response [47]. Several MAGE family members, including (MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, and MAGE-C2), were targeted in patients with melanoma using a peptide vaccine approach [48, 49]. Previously, recombinant vectors encoding CTA and short hairpin RNA were shown to help boost effective T cell response [50]. Among different CTA, NY-ESO-1 was found to be a decent applicant for cancer immunotherapy. Evidence shows that NY-ESO-1 possesses strong immunogenic potential, limited off-target toxicity, and thus has the potential to boost natural immune response [51, 52]. A variety of adjuvants were used to boost cytotoxic $CD8^+$ T lymphocyte action in response to

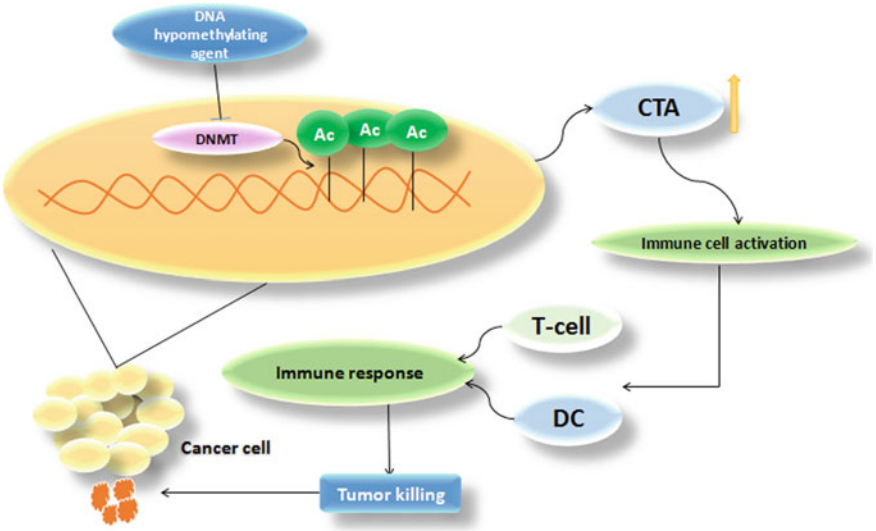

Fig. 2 Epigenetic basis of CTA expression and implication in immunotherapy

MHC class I-restricted peptides, such as granulocyte/macrophage colony-stimulating factor, montanide-ISA-51 (Montanide), polyinosinic–polycytidylic acid-stabilized by lysine and carboxymethyl cellulose (Poly-ICLC), incomplete Freund [53]. These adjuvants effectively induce humoral and cellular responses and are associated with a decrease in melanoma burden in mice [54]. Numerous clinical trials have investigated and elucidated NY-ESO-1 based T cell approach in hepatocellular carcinoma (NCT03175705), pancreatic cancer (NCT03192462), breast cancer (NCT03093350), synovial sarcoma (NCT03250325), haematological cancer (NCT02494167, NCT02291848) and other advanced solid tumors (NCT03047811, NCT02457650, NCT02869217, NCT02366546) [52].

6 Epigenetic Mediated CTA Expression in Immunotherapeutic Approach in Cancer

Epigenetic changes include alterations in histone modifications, DNA methylation, and non-coding RNAs associated with progression and development of metastasis, genome organization in cancer, and immune evasion [55, 56]. Epigenetic dysregulation plays a crucial role in the immunogenic deficiency of most cancer cells, culminating in the development of an immune-suppressive environment. Proof of concept studies has highlighted alterations in epigenetic signature associated with impairment in the phenotype of immune cells [57]. Several CTA acts as a potential

target for various epigenetic drugs. Reactivation of genes associated with epigenetic events can lead to new CTA expression. It has also been observed that both DNMT and HDAC inhibitors (DNMTi and HDACi) can upregulate the expression of MHC class I on the cell surface and lead to efficient antigen presentation [58]. Multiple lines of evidence suggest that treatment of HDACi and DNMTi can inhibit T cell exhaustion, activate chemokine repression, and increase tumor antigen expression, leading to the alteration of the immune-suppressive tumor microenvironment (TME) and increasing TIL [59]. Evidence shows that 5-AZA-CdR demethylates high molecular weight melanoma-associated antigens leading to the re-expression at RNA and protein levels (70). Constitutive expression of CTAs like MAGE-A3, PRAME, ROPN1, SCP-1, SLLP1, and SPO11 was found to be observed in several cancers [60]. Treatment with demethylating agents is associated with the individual expression of the above CTAs [61].

7 The Current Advancement in CTA Mediated Approach to Immunotherapy

Cancer immunotherapy has emerged as a successful treatment option for the various types of advanced cancers. New ways to block immune checkpoint regulators, overcome immunological tolerance, such as modified T cell treatment, or identify novel tumor antigens through next-generation sequencing have ushered in a new era of cancer immunotherapy [62]. Passive and active immunotherapy are both used in cancer immunotherapy. Active immunotherapy attempts to stimulate the self-immune system to attack tumor cells via vaccination, non-specific immunomodulation, or targeting specific antigen receptors. In contrast, passive immunotherapy shows the administration of agents such as mAbs, lymphocytes, or cytokines that enhance existing antitumor response [63].

The role of CAR T-cell therapy has shown to be of paramount importance for non-responders and patients with very few immunogenic tumors, like breast, pancreatic, and other hematological cancers [64]. T cells expressing TCR can be engineered to help recognize tumor-associated epitopes and generate therapeutic T cells with significant antitumor activity. Evidence from previous phase 1 and phase 2 clinical trials has discovered potential implications of T cell-based therapy targeting specific CTA in cancer. Evidence shows that NY-ESO-1 is a prominent target in multiple cancer types [65]. Better clinical response and survival rates were observed in melanoma and synovial sarcoma patients when adoptive T cell therapy with HLA-A2 limited NY-ESO-1/LAGE-1 transduced $CD8^+$ T cells was implicated [66].

Similarly, one more described CT antigen, PRAME, is being confirmed as a mark in several immunotherapeutic strategies. The intracellular nature of PRAME makes it challenging to be detected by the antibody, so an antibody was developed to mimic the TCR antibody with the same specificity [67, 68]. PRAME peptide was mainly

recognized, which are present at the complex on HLA-A2, and delivered proof-of-concept to antibodies to recognize and produce an immune response for intracellular antigens, which are only targetable with modified TCRs. In addition to this, the role of dendritic cells (DC) has shown to be highly promising in the clinical setting in the immunotherapeutic approach. DC is one of the most potent antigen-presenting cells (APC) and plays a vital role in regulating innate and adaptive immune responses [69]. Dendritic cell-based immunotherapy attempts to raise the number of competent DC (and therefore tumor-specific T cells) to change the balance from immunosuppression towards immune surveillance or reprogram the immune system from the 'escape' phase to the equilibrium or exclusion phase [70]. It has been observed that combining the DNMTi with CTA exact CAR-T cells can limit the heterogeneous expression of the antigen within the tumor; however, available antigen leak alternatives where without antigen cells are there in the tumor [71].

Therefore, to solve this obstacle, multiple antigen targeting approaches encompassing joint exact CAR T cells, bi-specific CAR T cells, and cycle CAR T cells show auspicious consequences in declining antigen leak and cumulative antitumor efficiency [71, 72].

Furthermore, past decades have witnessed the clinical benefit of the combinatorial therapeutic approach in cancer. This may include chemotherapy, blockade of immune checkpoints, cancer vaccine, radiation therapy with potential epigenetic modulators for CTA mediated by T cell or a DC-based approach will be highly promising to foster better clinical benefit [67, 73]. Clinical trials combining DAC and DC vaccination targeting MAGE-A1, MAGE-A3, and NY-ESO-1 have shown complete remission in recurrent neuroblastoma patients [74]. Identifying new potential CTA with tumor specificity, developing ideal adjuvants for efficient $CD8^{+}$T lymphocyte recombinant vectors encoding CTA and short hairpin RNA, multiple combinatorial treatment strategies, and multi-peptide mediated CTA approach will be highly promising in the clinical setting. To tackle the challenges associated with the CTA-mediated immunotherapy approach, identification, screening, and comprehensive construction of the CTA peptide library are quintessential for a better-personalized approach and anticipatory medicine.

8 Conclusion

Despite the remarkable clinical advancement, cancer is still a prominent malignancy. Immunotherapy is clinically beneficial in the clinical setting. The CTA-based immunotherapeutic approach proved to be patient-specific and therapeutically beneficial. Epigenetic treatment can augment CAR T cell-CTA mediated approach and has shown to be clinically effective. A future study must be carried out to explore the possible pros and cons of combinatorial treatment with an epigenetic mediated CTA approach which will upregulate CTA expression in MHC class I and class II is increased in tumor cells as well as co-stimulatory molecules by APC for a better clinical outcome (Table 1).

Table 1 Epigenetic target and their implication in CTA mediated approach in cancer

Epigenetic Players	Immunotherapy Players	Cancer type	Phase, Trial ID	References
Histone deacetylase inhibitors (target)	Nivolumab (PD-1)	Colorectal cancer	I/II, NCT03993626	[75]
	Avelumab (PD-L1)	GI cancer	II, NCT03812796	[76]
CXD101 (Pan HDAC)	Pembrolizumab (PD-1)	Bladder cancer	II, NCT03978624	[77]
Domatinostat (HDAC1,2,3)	Atezolizumab	Melanoma	II, NCT03765229	[78]
Entinostat (HDAC1,2,3)	Nivolumab (PD-1)	MDS	I, NCT02936752	[78]
Mocetinostat (Pan HDAC)	Aldesleukin (IL-2)	Metastatic uveal melanoma	II, NCT02697630	[78]
Tinostamustine (Pan HDAC)	Nivolumab (PD-1), Ipilimumab	Breast cancer	I/II, NCT03280563	[79]
Vorinostat (Pan HDAC)	(CTLA-4)	Cholangiocarcinoma, pancreatic	II, NCT03250273	[75]
Panobinostat	Durvalumab (PD-L1)	adenocarcinoma	I/II, NCT01038778	[75]
Givinostat	Nivolumab (PD-1)	Renal cell carcinoma	I, NCT02453620	[80]
Belinostat	Pembrolizumab (PD-1)	Breast cancer	I/II, NCT02805660	[75]
	Bortezomib (proteasome inhibitor)	NSCLC	I, NCT03903458	[81]
	plus dexamethasone	Melanoma	I, NCT03150329	[75]
	Chemo/ImmuAget	Lymphomas	I, NCT02619253	[82]
	NA	Renal cell carcinoma	I/II, NCT02638090	[82]
	Zidovudine (±IFNα-2b), pevonedistat	NSCLC	I/II, NCT02538510	[81]
	(NEDD8-activating enzyme inhibitor)	Head and neck	Phase I/III	[83]
	or temozolomide (chemotherapy) plus	Multiple myeloma	Phase II	[76]
	radiotherapy	Various solid carcinomas, lymphomas	Phase I/II	[84]
		and/or other haematological	Phase I, NCT03323034	[85]
		malignancies		[85]
		Chronic myeloproliferative neoplasms		
		T cell leukaemia or lymphoma, MDS		
		or AML, glioblastoma or various		
		other solid carcinomas and		
		haematological malignancies		

(continued)

Table 1 (continued)

Epigenetic Players	Immunotherapy Players	Cancer type	Phase, Trial ID	References
DNA methyltransferase inhibitors	Avelumab (PD-L1)	DLBCL	III, NCT02951156	[76]
Azacytidine	Alemtuzumab (CD52)	Myeloid malignancies	II, NCT02497404	[77]
Oral azacytidine (CC-486)	Pembrolizumab (PD-1)	AML	II, NCT02845297	[75]
Decitabine	Pembrolizumab (PD-1)	AML	II, NCT03769532	[75]
Guadecitabine	Pembrolizumab (PD-1)	Pancreatic cancer	II, NCT03264404	[75]
	Anti-PD-1 antibody	MDS	II, NCT03094637	[79]
	Dendritic cell vaccine (NY-ESO-1,	Ovarian cancer	II, NCT02900560	[75]
	MAGE- A1 MAGE-A3)	NSCLC	II, NCT02546986	[81]
	Atezolizumab (PD-L1) Durvalumab	Melanoma	II, NCT02816021	[75]
	(PD-L1) GVAX (Cell vaccine)	T cell lymphomas Lymphomas	II, NCT03240211	[82]
	Ipilimumab (CTLA-4)	AML	I, NCT03445858	[82]
	Pembrolizumab (PD-1)	Breast cancer	I, NCT03969446	[82]
		Solid tumors	II, NCT02957968	[75]
		Pediatric brain tumors	I/II, NCT02961101	[82]
		Urothelial carcinoma	I/II, NCT02332889	[82]
		Liver, pancreatic, bile duct,	II, NCT03179943	[75]
		gallbladder	I, NCT03257761	[75]
		Colon cancer	I, NCT01966289	[82]
		Melanoma	I, NCT02608437	[82]
		Ovarian	II, NCT02901899	[82]
		Prostate, NSCLC	I, NCT02998567	[86]
				[79]
				[75]
Histone modifications (target)	Pembrolizumab (PD-1) Ipilimumab	Bladder cancer	I/II, NCT03854474	[86]
Tazemetostat (EZH2) CPI-1205	(CTLA-4) Nivolumab (PD-1)	Solid tumors	I/II, NCT03525795	[86]
(EZH2)		Advanced tumors	I/II, NCT02419417	[86]
BMS-986158(BRD2/3/4, BRDT)				

(continued)

Table 1 (continued)

Epigenetic Players	Immunotherapy Players	Cancer type	Phase, Trial ID	References
Multiple combinations	Nivolumab	NSCLC	II, NCT01928576	[76]
Azacytidine, entinostat	Pembrolizumab	AML	II, NCT04284787	[83]
Azacytidine, venetoclax (Bcl-2)	Pembrolizumab	Metastatic solid tumors	I/II, NCT02959437	[69]
Azacytidine, epacadostat (IDO-1)	Pembrolizumab Pembrolizumab	Lung cancer	I, NCT03220477	[87]
Mocetinostat, guadecitabine	Pembrolizumab	Glioblastoma	I, NCT03426891	[87]
Vorinostat,temozolomide	Multiple agents	Breast cancer	II, NCT04190056	[87]
Vorinostat, tamoxifen	Pembrolizumab	Breast, prostrate, pancreas, AML	II, NCT02395627	[87]
Multiple agents	Tremelimumab	Colorectal cancer	I, NCT03878524	[87]
Azacytidine, romidepsin (Pan HDAC)	(CTLA-4) Durvalumab (PD-L1)	Head and neck cancer	I, NCT02512172	[84]
Azacytidine	Pembrolizumab	NSCLC	I/II, NCT03019003	[75]
Decitabine, tetrahydrouridine			I/II, NCT03233724	

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Epigenetics

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Abstract Cancer is one the most dreadful diseases in the world and its genetic origin has been undoubtedly proved and studied worldwide. Although, in recent decades, epigenetics has managed to shed light on many unknown mechanisms of carcinogenesis, being irrepressible by genetic laws. Epigenetic alterations represent a totality of heritable changes in gene expression that do not affect DNA sequence. Four major epigenetic alterations: DNA epigenetic alterations, histone post-translational modifications, remodeling complexes and non-coding RNAs are described in more detail in this chapter. In cancer, epigenetic deregulations are preceded by genetic mutations in genes of epigenetic machinery, and cause in result modifications of chromatin state. In this case, upregulation of oncogenes or downregulation of tumor suppressor gene expression became the worst outcome. In comparison with genetic mutations, epigenetic ones can be reversible. Therefore, different therapeutic strategies are developing to restore normal epigenetic landscape in tumor cells, pharmaceutical agents being classified by their main epigenetic targets. Common epigenetic regulators, such as HDAC inhibitors, DOT1L inhibitors, LSD inhibitors, EZH2 inhibitors and others are also described below. Besides of natural or synthetic regulators, epigenetic modifications can also be triggered by predisposition to different health conditions, onset of other non-cancerous diseases, virosis, aging or stress. Another background by which epigenetic profile is affected, and therefore can be reversed, includes different lifestyle factors such as environmental circumstances, diet or practicing exercises. For these reasons, we hope that this chapter will highlight the importance of epigenetic deregulations in cancer, and will also encourage further investigations of epigenetic mechanisms, validation of novel epigenetic biomarkers as well as development of new suitable epigenetic drug regulators in order to improve cancer therapy.

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1 Carcinogenesis

Nowadays, cancer is one of the most frightening diseases, characterized by the uncontrolled growth of abnormal cells. Unfortunately, its power over human lives continues to lie in a variety of well-orchestrated and precise dysregulations of important intra- and intercellular pathways that control vital cellular processes such as nutrition, growth, proliferation, survival, and intercellular communication, and mortality. Once these dysregulations have been initiated, they are difficult to control because even the slightest trigger factor—roots its effects into a vast network of interconnected cascades [1, 2]. In defiance of the fact that clinicians are not yet able to completely eradicate them, cancer is becoming more and more vulnerable due to all the revealing information which we have continued to accumulate since the understandings of the first basic tumorigenesis mechanisms were outlined. In 2000, Hanahan and Weinberg defined these driving forces as hallmarks of cancer, where six essential mechanisms acquired by tumor cells were described: self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion, and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis [1]. These hallmarks had been instantly acknowledged in cancer's existing status as a "genetic" disease and a plethora of studies have been accomplished to demonstrate and strengthen this concept. Numerous genetic mutations have been identified and associated with a malignant profile, being used therefore as biomarkers for distinguishing normal versus cancerous tissues [2, 3], as well as different tumor types and even different stages of disease within the same cancer type [4]. However, the general trait of any malignant cell has been established as genomic instability, prone to aberrant and abnormal survival. Therefore, such alterations as deletions, substitutions, or translocations always led, in one way or another, either to the activation or overexpression of oncogenes, such as *MYC, RAS, RAF, AKT, BCL-XL, BCL-2*, or to the inactivation or silencing of tumor suppressor genes such as *P53, pRB, BAX, BAK*. The first ones favored proliferation, invasion, metastasis, expression of growth factors, and angiogenic or antiapoptotic signals, conversely acting the last one's [2–4].

Even though cancer development may have a genetic origin has been undoubtedly approved, there were still some discrepancies in terms of attempts to associate genotype with phenotype profile. Consequently, the studies of cancer mechanisms have continued so that at present, the data in the literature already list 14 hallmarks of cancer. The last 4 hallmarks, described with examples by Hanahan in 2022, are very different from the original ones and open much more horizons for a better understanding of complex tumor mechanisms than the first six originally described. One of these, which raise particularly increased interest in bringing to light the concept

of the genetic-independent evolution of cancer, is termed "nonmutational epigenetic reprogramming" [3, 5].

2 Major Epigenetic Alterations in Cancer

Epigenetic alterations are in general related to normal biological processes such as aging or differentiation, being considered a reversible process. Alteration of epigenetic signatures by a wide range of factors (particularly environmental), along with genetic and transcriptomic alterations, are considered as driving events in several diseases including cancer [6]. Therefore, the identification of tumor-specific epigenetics and the factors that affect epigenetic patterns should be evaluated to unmask truly disease-specific alterations.

Epigenetics, which means "upon the genes", represents the study of heritable changes in gene expression, that do not involve alterations in the DNA sequence, consequently involving phenotype changes without the genotype ones even in the cells which share identical genome. The term "epigenetics" appeared for the first time in 1942 when Conrad Waddington coined it to describe phenomena that do not follow "normal genetic rules" [7].

Epigenetic changes play an essential role in a series of normal biological processes, such as embryonic development, viral protection, genetic imprinting, and X-chromosome inactivation [8]; disruption of these epigenetic processes has been considered key mechanisms in a variety of pathologies including Beckwith-Wiedemann (BWS), Silver-Russell, Prader-Willi and Angelman syndromes as well as autoimmune diseases—systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), Sjogren's syndrome (SS), autoimmune thyroid diseases (AITD) and different neurological diseases [9–11].

Although epigenetic reprogramming is officially assigned as the hallmark of cancer just recently, as mentioned above, having an important role in growth-related pathways gave the earliest clues about the connection of these two about 40 years ago. In 1983, Feinberg and Vogelstein enlightened the first epigenetic mechanism attributed to cancer cells. Already knowing that the methylation process is related to the silencing of certain genes in some pathologies, they used Southern blotting and methylation-sensitive enzymatic restriction to demonstrate that the genome of cancer cells is hypomethylated at CpG dinucleotides compared to normal cells, which have generally hypermethylated genome [12, 13]. Other milestones in the history of human cancer epigenetics can be found comprehensively described by Feinberg and Tycko in 2004 [14].

Initially, three main types of epigenetic mechanisms were distinguished: DNA methylation, loss of imprinting (LOI), and histone modifications [14]. Although they had already encompassed a significant amount of information regarding the most important epigenetic elements, nowadays four categories of epigenetic mechanisms have been established with more well-clarified mechanisms within one category. These epigenetic modifications include:

(1) DNA epigenetic alterations,
(2) histone post-translational modifications (PTMs),
(3) remodeling chromatin complexes, and
(4) noncoding RNAs regulation [10, 14–17], schematic representation presented in Fig. 1.

All four epigenetic mechanisms determine either gene expression or silencing by controlling the chromatin state (condensed or decondensed). This is achieved through differential expression of enzymes that have abilities to change the accessibility of chromatin for binding of DNA transcription factors. A common way to identify and distinguish these enzymes is to assign them one of the statuses "writer", "reader" or

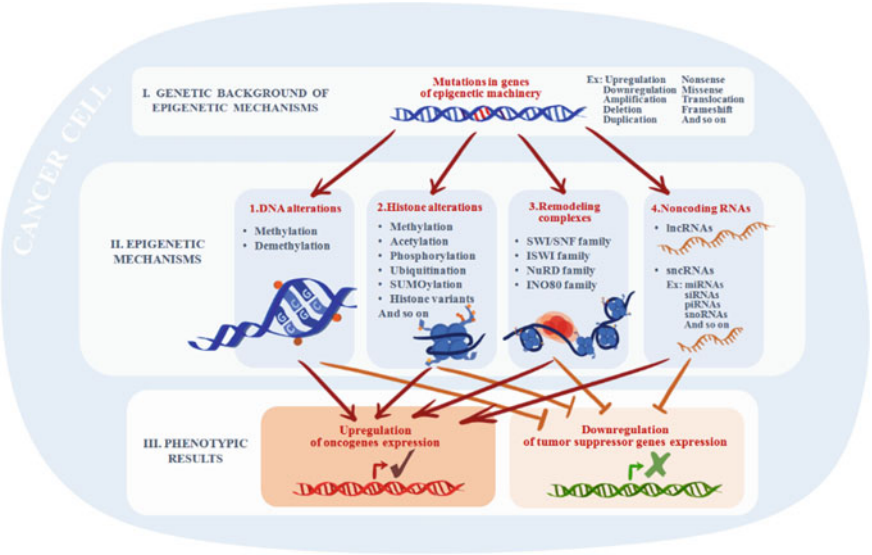

Fig. 1 Schematic representation of three main key steps of epigenetic regulation in cancer cells. **I. Genetic background of epigenetic mechanisms**. Initially, genetic mutations of certain genes that are involved in the functioning of the epigenetic machinery occur. **II. Epigenetic mechanisms**. Endpoint products of these altered genes, being either enzymes, protein complexes, or RNAs, become under- or overexpressed and lead to epigenetic changes which include DNA (1) and histone (2) alterations, aberrant functioning of remodeling complexes (3), and noncoding RNAs (4). **III. Phenotypic results.** Acting separately or in combination with each other, epigenetic changes specifically modulate the accessibility of promoters for binding with transcription factors thus regulating the expression of oncogenes or tumor suppressor genes. Aberrant expression of these target genes induces dysregulations in pathways of growth, proliferation, survival, invasion, apoptosis, and senescence, which play a crucial role in tumorigenesis and cancer development. *Abbreviations* SUMO—Small Ubiquitin-like Modifier; SWI/SNF—switching defective/sucrose non-fermenting; ISWI—imitation-switch; NuRD—nucleosome remodeling and histone deacetylase; INO80—inositol 80; lncRNAs—long noncoding RNAs; sncRNAs—small noncoding RNAs; miRNAs—microRNAs; siRNAs—small small interfering RNAs; piRNAs—PIWI-interacting RNAs; snoRNAs—small nucleolar RNAs

"eraser". The enzymes which deposit such modifications by adding different chemical groups are called "writers". They predominantly fall into the first two categories, contributing to DNA or histone modifications. The molecular marks which recruit chromatin remodelers and noncoding RNAs to mediate downstream effects, their interaction is controlled by proteins called 'readers'. Additionally, chemical modifications can be removed by 'erasers', a fact that can label epigenetic alterations as reversible ones [18]. Currently, all four categories of epigenetic mechanisms may be involved, separately or in combination with each other, in the development of cancer. Further, the main mechanisms from each category are briefly discussed. Some representative examples are presented in Table 1.

2.1 DNA Epigenetic Alterations

DNA epigenetic alterations include methylation, demethylation, hydroxymethylation, and its oxidation derivatives. The concurrence of DNA methylation and demethylation is in general related to transcription regulation. For DNA methylation an important role is attributed to DNA methyltransferases (DNMT) and for demethylation to the ten-eleven translocation (TET) [19].

The first one is best-studied and refers to the addition of a methyl group (–CH3) at the fifth position on the pyrimidine ring of cytosine (5mC) within CpG dinucleotides. These are clustered together into "CpG islands" and are found in about 40–60% of human gene promoters and repetitive regions of the DNA [20]. Two aberrant forms of DNA methylation can be distinguished during tumor development and progression: global DNA hypomethylation and local hypermethylation. The first one implies an overall loss of 5-methyl-cytosine. The last one makes promoters inaccessible for binding with transcription factors (TFs) and therefore it is a significant mark of gene silencing associated with gene inactivation. In cancer, particularly tumor suppressor genes and certain regulatory antioncogenes have a hypermethylated promoter, that leads to important alterations responsible for the tumorigenesis [21]. The process of methylation is catalyzed by DNA methyltransferases (DNMTs) which transfer a methyl group from donor S-adenosyl-L-methionine (SAM) to the cytosine residue. There are three main types of DNMTs known in mammals: DNMT1, DNMT3a, and DNMT3b, each having a specific mechanism of action. DNMT1 maintains the existing methylation pattern following DNA replication, while DNMT3a and DNMT3b catalyze *din novo* unmethylated CpGs, especially at new-formed strands of DNA after replication. Another family member, DNMT-3L lacks intrinsic methyltransferase activity, instead, it interacts with DNMT3a and DNMT3b to facilitate the methylation of retrotransposons [22]. In addition to direct inhibition of gene expression, methylated sites can also recruit specific proteins from the methyl-binding domain (MBD) family, such as MBD1, MBD2, MBD3, and MBD4, which in their turn recruit histone-modifying enzymes and chromatin-remodeling complexes [23, 24]. This MBD1 inhibition in pancreatic cancer affected the antioxidant response element target genes through epigenetic regulation of KEAP1 [25]. MBD1 plays a

Table 1 Cancer mutations and expression levels affecting epigenetic mechanisms

Category of epigenetic mechanism (most representative)	Altered genes and type of mutation (most representative)	Gene function/role	Cancer type	References
DNA modifications				
DNA methylation	DNMT1↑	Maintenance of methylation, gene silencing	Colorectal cancer, ovarian cancer, hepatocellular carcinomas, prostate cancer	[69–72]
	DNMT3a↑, mutant	De novo methylation	Acute myeloid leukemia, myelodysplastic syndrome, prostate cancer	[72–74]
	DNMT3b↑	De novo methylation	Ovarian cancer, colorectal cancer, prostate cancer	[70–72]
	DNMT3L↑	Methylation through interaction with DNMT3a and DNMT3b	Prostate cancer, cervical cancer	[72, 75]
DNA demethylation/hydroxymethylation	TET1↑	Dioxygenase, convert 5mc into 5hmc, oncogenic role	Lung cancer	[76]
	TET1↓	Dioxygenase, convert 5mc into 5hmc, tumor suppressor role	Colon cancer, breast cancer, gastric cancer	[29, 77–79]
	TET2↓, mutant	Dioxygenase, convert 5mc into 5hmc, tumor suppressor gene	Non-Hodgkin lymphoma, myelodysplastic syndrome, chronic myelomonocytic leukemia, acute myeloid leukemia	[80, 81]
	TET3↑	Dioxygenase, convert 5mc into 5hmc, oncogenic role	Ovarian cancer	[82]

(continued)

Table 1 (continued)

Category of epigenetic mechanism (most representative)	Altered genes and type of mutation (most representative)	Gene function/role	Cancer type	References
Metyl-CpG-binding proteins	MBD1↑	Transcription repression, epigenetic regulation of KEAP gene	Pancreatic cancer	[25]
	MBD1↓	Transcription repression	Colorectal cancer	[83]
	Kaiso↑	Transcriptional regulator with bimodal DNA-binding specificity; signal transduction and cell adhesion molecule	Lung cancer	[27, 84]
Histone post-translational modifications				
Histone methylation	MLL1↑ (KMT2A)	Transmethylation	Breast cancer	[85]
	MLL2↑ (KMT2D)	Lysine N-Methyltransferase	Colon cancer, breast cancer	[85]
	MLL3, mutant (KMT2C)	Lysine methyltransferase, affect transcriptomic pattern	Colorectal cancer, prostate cancer	[86–88]
	EZH2↑	Lysine N-Methyltransferase, PCR2 subunit	Prostate cancer, lung cancer	[89, 90]
Histone demethylation	JHDM1A↑ (KDM2A)	Lysine demethylase	Osteosarcoma, ovarian cancer, breast cancer, lung cancer	[91–93]
	LSD1↑	Lysine-specific demethylase; promote carcinogenesis	Cervical cancer, prostate cancer, lung cancer, colorectal cancer, acute myeloid leukemia, bladder carcinomas	[94–96]

(continued)

Table 1 (continued)

Category of epigenetic mechanism (most representative)	Altered genes and type of mutation (most representative)	Gene function/role	Cancer type	References
Histone acetylation	HAT1↑	Histone acetyltransferase 1	Esophageal carcinoma, Pancreatic cancer	[97, 98]
	HAT1↓	Histone Acetyltransferase 1, promote apoptosis	Lung cancer, osteosarcoma	[99]
	p300↓	Transcription regulation via chromatin remodeling; increase protein instability	Colorectal cancer, multiple cancers	[100, 101]
	p300↑	Acetyltransferase, transcriptional co-activator protein; drug resistance	Pancreatic cancer, squamous cell carcinoma, colorectal cancer, hepatocellular carcinomas, lung cancer	[102–106]
	GCN5↑	Lysine acetyltransferase, oncogenic role; drug resistance	Breast cancer, lung cancer, colon cancer	[107–109]
Histone deacetylation	HDAC1↑	Histone deacetylation, gene repression	Ovarian cancer, lung cancer, gastric cancer, breast cancer	[70, 110–112]
	HDAC2 ↑	Histone deacetylation gene repression	Ovarian cancer, lung cancer	[70, 110]
	HDAC3↑	Histone deacetylation Gene regulation	Acute myeloid leukemia, lung cancer	[110, 113]
Histone variants	mH2A2↓	Core histone protein, tumor suppressor role	Malignant melanoma	[41]
	H2A.Z.2↑	Histone protein, oncogenic role	Malignant melanoma	[42]

(continued)

Table 1 (continued)

Category of epigenetic mechanism (most representative)	Altered genes and type of mutation (most representative)	Gene function/role	Cancer type	References
	mH2A1.1↓	Core histone protein, tumor suppressor role	Colorectal cancer	[43]
	mH2A1.2↑	Core histone protein, oncogenic role	Colorectal cancer	[43]
Chromatin remodeling complexes				
SWI/SNF complexes	BRG1↓ (SMARCA4)	Catalytic atpase and helicase subunit	Lung cancer	[47, 114]
	BRG1↑ (SMARCA4)	Catalytic atpase and helicase subunit, regulated transcription of cell proliferation and cell cycle related genes	Colorectal cancer, breast cancer	[48–50]
INO80 complexes	ARID1A↓, mutation	Variant subunit, BAF complex only; mediate endocrine resistance to therapy or metastasis	Breast cancer, gastric cancer, colorectal cancer	[115–117]
	SMARCA2↓	Catalytic atpase subunit	Lung cancer	[118]

Abbreviation ↓ downregulation ↑ upregulation

role in tumorigenesis by repressing tumor suppressor genes like *CDH1, RASSF1A, TIMP3, P14ARF*, and *Rb* [26].

Somatic mutations in DNMTs and MBD proteins have been associated with deregulated pathways in different cancers, having oncogenic or tumor suppressor roles [27]. As effect of MBD epigenetic gene silencing mechanisms a wide range of transcriptional factors are released (Table 1).

In contrast to DNMTs, the ten-eleven translocation (TET 1–3) family of proteins catalyzes the oxidation of 5mC into 5-hydroxymethylcytosine (5hmC) and further products which modulate the DNA methylation landscape. TETs proteins have two cofactors, Fe(II) and 2-oxoglutarate (2OG), which are indispensable for successively 5mC oxidation. TETs loss-of-function is commonly observed in various cancers [28]. DNA hydroxymethylation facilitated by TET1 controlling the WNT signaling is a key factor in the tumor growth [29]. TETs' role in cancer is considered as context-dependent tumor-suppressor genes and/or oncogenes in solid tumors [30]. The anti- or oncogenic roles are directly related to the combination of different signaling pathways in different tumors [31].

2.2 Histone Post-translational Modification

Another type of epigenetic alteration occurs at the chromatin level. Here, nucleosome forms the basic structural unit of chromatin. It consists of DNA wrapped around an octamer of histone proteins which are represented by two copies of proteins H2A, H2B, H3, and H4. Histone modifications can contribute to chromatin compaction, nucleosome dynamics, and transcription alteration regulated by a fine-tuning mechanism regulated by chromatin modifiers and histone modifications [6, 32].

Histones are characterized by N-terminal tails rich in positively charged lysine (K) which in combination with a negatively charged DNA backbone confer a tightly packed state of chromatin [33]. Epigenetic changes occur when histones undergo post-translational modifications (PTMs) which primarily involve the addition or removal of certain chemical groups by specific enzymes at their N-terminal tails. These modifications trigger conformational changes in the chromatin structure, conferring either condensed (heterochromatin) or relaxed (euchromatin) state. Tight nucleosomes can become loose when the positive lysine residues are neutralized, therefore the access of the transcriptional machinery to the adjacent promoter of the gene will be enabled and the gene will be expressed. Conversely, the addition of more positive residues or groups to the surface of histones can enforce a chromatin tightened state and increase gene repression, without involving any DNA alterations in both cases. Consequently, more types of PTMs can be distinguished depending on which chemical group was added. The most well-known modifications are methylation, acetylation, and phosphorylation. These modifications are also established as epigenetic histone marks.

2.3 Histone Methylation

Histone methylation is catalyzed by writer-enzymes, histone methyltransferases (HMTs). It typically includes the addition of methyl groups (–CH3) to lysine (K) and/or arginine (R) residues. Finale regulation (activation or repression of the gene) results not only from the process of methylation per se but also from the position of methylated amino acid and the number of methyl groups added. For example, one of the most recognizable histone marks is the addition of three methyl groups by lysine methyltransferases (KMTs) at lysine 9 of histone H3 (H3K9me3) which results in gene silencing. Meanwhile, mono- and dimethylation of the same residue (H3K9me and H3K9me2) has the opposite effect. Other activating and repressive marks are H3K4me3 and H3K27me3, respectively [34]. One specific methyltransferase is enhancer of zeste homolog 2 (EZH2), which is a catalytic component of the polycomb repressive complex 2 (PRC2) and plays the primary role in the trimethylation of H3K27. Therefore, it is one of the important epigenetic elements which is responsible for gene silencing, being usually overexpressed in cancers [35]. Removal of the methyl group is performed by histone demethylases (HDMs). The expression level of both HMTs and HDMs can be altered in different tumor types, more information is provided in Table 1.

2.4 Histone Acetylation

Histone acetylation is catalyzed by histone acetyltransferases (HATs) which add acetyl group (–CH3CO) and neutralize the positively charged histone thus leading to conformational changing in chromatin structure and activation of gene transcription. H3K27ac and H2BK5ac are some examples of histone marks that correspond with actively transcribed genes. Histone deacetylases (HDACs) act conversely, thus inducing a back shift to the repression state of the gene. HDACs bind to and deacetylate a diversity of protein targets including transcription factors, involved in the control of cell growth, differentiation, and apoptosis [36]. A particular type of HDACs is a highly conserved family of NAD(+)-dependent HDACs called sirtuins (SIRTs). Seven mammalian sirtuins (SIRT1–7) are known to be implicated in many cellular processes, especially in epithelial-mesenchymal transition (EMT), invasion, and metastases [37].

2.5 Histone Phosphorylation

Histone phosphorylation, performed by kinases, occurs mainly at serine (S), threonine (T), and tyrosine (Y) residues and is associated with accessible chromatin conformation. Many histone marks have been found mutually working together,

for example, histone H3 phosphorylation at tyrosine41 (H3Y41) is enriched at active promoters close to transcription start-sites (TSS) together with the H3K4me3 mark [38], loss of the trimethylation of H4K20 (H4K20me3) and acetylation of H4K16 (H4K16Ac), along with DNA hypomethylation is labeled as the common hallmark of primary tumors [39] as well as reduced levels of lysine acetylation (H3K9ac, H3K18ac, H4K12ac) and methylation (H3K4me2, H4K20me3) and arginine methylation (H4R3me2) [39]. Other PTMs include ubiquitination, SUMO (small ubiquitin-like modifiers)-ylation, neddylation citrullination, deamination, formylation, biotinylation, O-GlcNAcylation, propionylation, butyrylation, crotonylation, proline isomerization, ADP-ribosylation and lactylation [32, 34].

Besides covalent histone modifications which affect directly the state of chromatin, some alterations involve the exchange of canonical histones in the nucleosome with histone variants. Histone variants arise from mutations in genes that encode histone proteins. They became considered potential drivers of cancer initiations, being either up or downregulated in different cancer types [40]. For instance, macroH2A (mH2A) is one of the most distinguishable known histone variants, due to its special *macro* domain with the 25-kDa-sized globular module. In malignant melanoma, mH2A2 turned out to be the downregulated [41]. In contrast with mH2A, overexpression of variant histone H2A.Z.2 isoform presented an oncogenic role and provided a proliferative effect in the same malignance [42]. Other studies showed that even different splice isoforms can discriminate between different stages of tumor development and show differential expression levels, such as maH2A1.1 which is downregulated in primary colorectal cancer samples compared to normal colon tissue, while mH2A1.2 is upregulated [43].

2.6 Remodeling Complexes

While PTMs of histone represent intrinsic epigenetic changes at the chromatin level, there is also an extrinsic way to manipulate the chromatin state which is performed by remodeling complexes. Chromatin remodeling is considered an important gateway to regulating gene transcription. Therefore, this mechanism has important implications for targeted cancer therapeutic strategies, considering that cancer can select a multi-subunit remodeler proteome for oncogenic advantage [44]. Based on the different structures and enzymatic activity of these complexes, they are categorized into four major families: the switching defective/sucrose non-fermenting (SWI/SNF) family, the imitation-switch (ISWI) family, the nucleosome remodeling and histone deacetylase complex (NuRD), and the inositol 80 (INO80) families. Remodeling occurs when the interaction between DNA and histone proteins is reconfigured by specific ATP-dependent enzymes which make up the subunits of remodelers [45]. As result, remodelers can manipulate nucleosome sliding along DNA, create access to transcription factors to gene promoters, and eject or replace certain histone variants [46]. Families also have different domain structures, such as SANT domains, bromodomains, PHD domains, DNA-binding domains, and chromo-domains that

assign them certain specificity. Having a pivotal role in transcriptional profile regulation, mutations in these remodeling complexes were immediately associated with cancer malignancies. The most studied is SWI/SNF complex. It activates predominantly in two forms, based on its constituent core subunits: BRG1-associated factors (BAF) and polybromo-associated BAF (PBAF). The first one, as results from the name, contains subunits as BRG1 or BRM, and ARID1A/ARID1B, while the last one contains BRG1 only, and ARID2 and BRD7. SWI/SNF complexes have been found to function close to promoter or enhancer regions and interact with transcription to modulate gene expression and contribute to lineage specification, differentiation, and development. Consequently, it has been recognized as tumor suppressor complexes [47], although recent data accumulate controversial evidence [48–50], most of the studies being related to the regulation of mitotic cell divisions and DNA repair mechanisms [49–51]. BRG1 is considered not only a prognostic marker but also a therapeutic target [50, 52]. Generally, genes that encode component subunits of BAF or PBAF are found to be mutated, especially downregulated or inactivated in a variety of cancers [47, 52]. In contrast with SWI/SNF complexes, elements of other complexes, such as INO80, have been elevated in some cancer types mediating oncogenic signaling and promoting tumor growth [52–55].

2.7 Noncoding RNAs

Noncoding RNAs took a step forward in the overall regulation of gene expression, interfering before transcription and translation levels as well. Varieties of noncoding RNAs are categorized into two major types, according to their length: small noncoding RNAs (sncRNAs, under 200 nucleotides) and long noncoding RNAs (lncRNAs, more than 200 nucleotides) [56–58]. The sncRNAs include small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and microRNAs (miRNAs), the last ones being the most studied and strongly correlated with cancer development. These non-coding RNA transcripts regulate gene expression via complementarily binding to the 3′ UTR of the target mRNA [16]. Several studies revealed important aspects of epigenetics directly connected to this noncoding RNA transcript, particularly small RNAs that can direct the cytosine methylation and histone modifications that are involved in gene expression regulation [59].

The length of miRNAs represents approximately 22 nucleotides. Their role lies in complementary binding to a specific sequence of target mRNA and thus inducing mRNA-silencing. Therefore, many miRNAs have been found overexpressed in different tumor types, primarily downregulating the expression of tumor suppressor genes [23, 58, 60]. Nevertheless, some studies correlate the function of specific miRNAs with tumor suppressor activity [57, 58]. Differential expression of miRNAs is strongly correlated with the epigenetic process of DNA methylation due to the specific location of miRNA-encoding genes associated with CpG islands in their promoter regions. Additionally, miRNA genes might be located in

specific chromatin structures that predispose them even more to DNA methylation [61, 62]. For example, miR-200, which targets zinc finger transcription factor ZEB1 together with zinc finger homeobox protein ZEB2 and provides an inhibitory effect on the epithelial-mesenchymal transition (EMT) process, is subject to methylation and also trimethylation of H3K27 to favor EMT and promote cancer development [63] (Fig. 2a).

On the side of sncRNAs, lncRNAs encompass even more regulatory functions. They have been associated with modulation of mRNA processing, control of transcription in *cis* or *trans*, as well as of post-transcriptional process and protein activity, organization of nuclear domains, and interfering with chromatin remodeling complexes [58, 64]. Likewise, they have been positively correlated with both tumor suppression and tumorigenesis, being, therefore, up- and downregulated in a variety of cancers [58]. As aforementioned, lncRNAs can modulate epigenetic processes in multiple interconnected ways. For example, in prostate cancer—prostate cancer

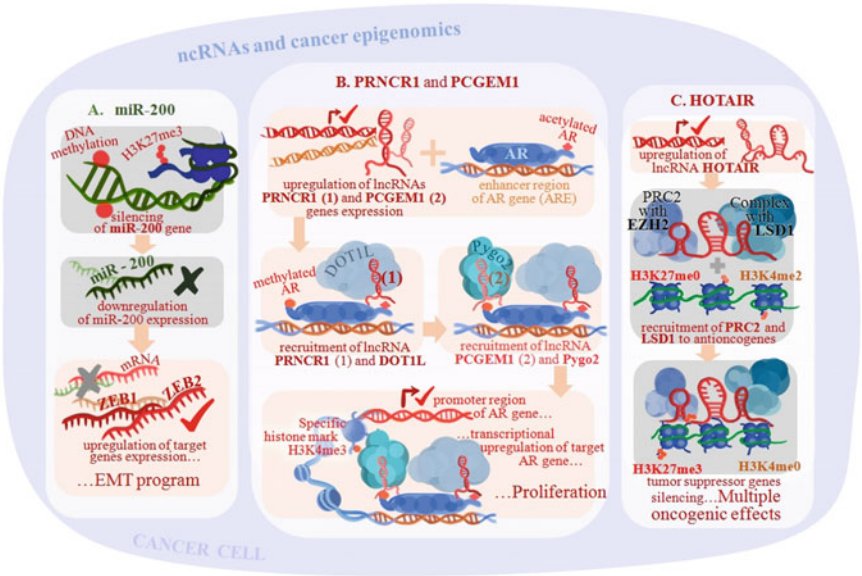

Fig. 2 Representative examples of ncRNAs that are involved in gene expression modulation through interaction with cancer epigenomics. **a** miR-200; **b** lncRNAs PRNCR1 and PCGEM1; **c** lncRNA HOTAIR. *Abbreviations* mRNA—messenger RNA; ZEB1—zinc finger transcription factor; ZEB2—zinc finger homeobox protein; EMT—epithelial-mesenchymal transition; PRNCR1—prostate cancer noncoding RNA1; PCGEM1—prostate cancer gene expression marker 1; AR—androgen receptor; ARE—AR response element; DOT1L—disruptor of telomeric silencing 1-like; Pygo2—Pygopus2; HOTAIR—HOX Transcript Antisense Intergenic RNA; PRC2—polycomb repressive complex 2; EZH2—enhancer of zeste homolog 2; LSD1—lysine-specific demethylase 1; H3K27me3—trimethylated lysine 27 of histone 3; H3K27me0—unmethylated lysine 27 of histone 3; H3K4me2—dimethylated lysine 4 of histone 3; H3K4me0—unmethylated lysine 4 of histone 3

noncoding RNA1 (PRNCR1) binds to the acetylated enhancer of androgen receptor and recruits histone H3K79 methyltransferase—disruptor of telomeric silencing 1-like (DOT1L). Consequently, methylation of androgen receptor facilitates the recruitment of another lncRNA, prostate cancer gene expression marker 1 (PCGEM1), to its N-terminal region. PCGEM1-recruited Pygopus2 (Pygo2) recognizes histone mark H3K4me3 and provide selective looping of enhancer with promoter, thus modulating gene expression of target gene androgen receptor (AR). AR enhance G1–S progression of cell cycle and therefore cell proliferation [65, 66] (Fig. 2b). In breast cancer, as well as in a variety of other cancers, well-known and overexpressed lncRNA HOX Transcript Antisense Intergenic RNA (HOTAIR) interacts with polycomb repressive complex (PRC2) and lysine-specific demethylase 1 (LSD1) thus recruiting them to the target gene and inducing gene silencing via H3K27-methylation and H3K4-demethylation [67] (Fig. 2c).

Different noncoding RNAs can also synergistically or antagonistically interact one with another to modulate gene expression. lncRNAs and circRNAs might act as miRNAs sponges by directly binding to them and abolishing their function. A study on breast cancer identified the lncRNA FAM83H-AS1 secludes miR-136-5p and therefore encourages metadherin-induced proliferation, migration, and invasion [68].

3 Epigenetics Drugs for Cancer Therapy

Considering the important function of the epigenetic dysregulation towards the origin and progression of cancer is considered an important cancer hallmark, an important number of preclinical and clinical studies are involved in testing and validation as a therapeutic strategy to restore the reversible normal epigenetic landscape in cancer cells by inhibiting enzymes of the epigenetic machinery in a wide range of cancer types [17, 119]. Until present, a wide range of natural or synthetic chemical agents as epigenetic regulators are tested and classified based on the main epigenetic target, the common DNMT inhibitors, HDAC inhibitors, DOT1L inhibitors, LSD inhibitors [120], EZH2 inhibitors, BET inhibitors [17]. Some of these inhibitors have been approved by the US FDA for the treatment of diverse malignancies and an important number of these compounds are undergoing clinical trials.

Inhibitors of *DNMT* and *histone acetyltransferases/deacetylases* have been revealed to inhibit tumor growth by reactivating epigenetically silenced tumor suppressor genes and silencing oncogenes [121].

DNMTs inhibitors can reverse the DNA hypermethylation status of tumor suppressor genes, they have been divided into two classes cytosine analog inhibitors and non-nucleotide analog inhibitors [16]. The most common demethylating are 5-azacytidine and 5-aza-2′-deoxycytidine, already approved for cancer therapy and hematologic pathologies. The main issue related to this type of agent is related to the unspecific reactivation of methylated sequences of tumor suppressor genes

CpG islands. In parallel, also a global genomic demethylation process that causes chromosomal instability was observed [24].

MBDs are considered a valuable target for cancer inhibition, to avoid problems related to genomic instability, by inhibiting DNA methylation per se [24].

HDAC proteins are related to multiple oncogenic steps, HDAC inhibitors are involved in the prevention of tumor suppressor genes if recruited to promoters together with fusion oncogenes such as *PML-RAR*α. Another application of HDAC inhibitors is to prevent the expression of HDACs proteins, that are in general overexpressed in multiple solid tumors or hematological malignancies, with high expression levels being in general related to an unfavorable prognostic [122].

Several *HDACi* are already approved in the clinic (Vorinostat, romidepsin, panobinostat, and belinostat in hematological malignancies), meanwhile others are tested currently in phase I or II clinical trials including pracinostat, givinostat, resminostat, abexinostat, entinostat, quisinostat [16].

Vorinostat and romidepsin were the first drugs to be approved that influence epigenetic post-translational modification of histone proteins [123]. Suberoylanilide hydroxamic acid (SAHA; vorinostat) is a non-selective broad-spectrum HDACI that induces acetylation of histones, this was demonstrated to be relegated with the overexpression of p21 as the effect of activation of the acetylated histone H3 and H4 in bladder carcinoma and endometrial stromal sarcomas [16, 124]. MS-275 inhibits HDACs 1–3 and 9, generally, this inhibitor was tested in conjunction with other agents [125]. Generally, this class of compounds is used to impede oncogenesis by acting apoptosis and cell cycle arrest and affecting the DNA damage pathway [122].

Preclinical work with BET inhibitors was focused on the comprehension of the relationship of BET proteins in regulating the cell cycle [122]. BET inhibition is generally related to transcriptional repression and cell cycle arrest [122]. Transcription factors are implicated in a wide range of pathologies, in a large number of human diseases such as cancers [126]. Accumulating investigations reveal that repression of EZH2 by small molecular inhibitors or gene knockdown leads to a decreased cell proliferation and tumor formation capacity [127] (Table 2).

4 How Epigenetic Processes Can Be Manipulated for Cancer Patient Benefit

The epigenetic processes are composed of DNA methylation, chromatin remodeling, histone modification, and non-coding RNA regulation [148–150]. These processes have a significant role in genome function and are dynamic, which means that they can be modified during their whole life by different factors [151, 152]. Because epigenetic processes are reversible, different factors can be used for epigenetic manipulation that can be implemented in cancer research. Epigenetic changes take place during our whole life and some epigenetic changes can be transferred from generation to generation [153]. Another important fact is that environmental conditions affect the

Table 2 Relationship between drugs and epigenetic mechanisms

Epigenetic mechanism	Altered expression	DRUG	Disease	Observation	References
DNMT	DNMTs↓	5-Aza-2-deoxycytidine	Myelodysplastic syndrome	Reverse the DNA hypermethylation status of tumor suppressor genes	[16, 128]
	DNMT1↓	Zebularine	Bladder carcinoma	DNA demethylating agent reactivate an epigenetically silenced gene	[129]
	DNMT1↓	MG98a	Renal carcinoma	Methylate DNA, local chromatin condensation and consequent repression of gene expression; reducing tumor progression	[130, 131]
	DNMT1↓	RG108	Colon cancer	Demethylation and reactivation of tumor suppressor genes	[15]
	DNMT1↓	Decitabine	Acute myeloid leukemia; myelodysplastic syndrome	–	[128]
HDAC	HDAC2, HDAC3, HDAC7	SAHA	Bladder carcinoma and endometrial stromal sarcomas	Antitumoral effect via p21 regulation and	[16, 124]
	HDAC1 and HDAC3↓	Valproic and acid RGFP966	**Acute myeloid leukemia**	**Reversal of chemoresistance in via DNA damage response**	[113]

(continued)

Table 2 (continued)

Epigenetic mechanism	Altered expression	DRUG	Disease	Observation	References
	HDAC3 and HDAC4↓	Valproic acid	Myocardial infarctions	Stimulatory effect on vascular endothelial tissue-type plasminogen activator expression	[132]
	HDACI↓, MS275↓	Entinostat (MS-275)	Malignant ascites	Inhibit malignant ascites development and tumor growth	[133]
	HDACi↓	Panobinostat	Multiple myeloma, pancreatic cancer	Relapsed and refractory disease	[134, 135]
EZH2		Quinoline derivatives	Solid tumors	Inhibition of EZH2 by evaluation of the H3K27 methylation	[136]
		GNA022	Human head and neck and breast cancer cell lines	Specifically, and covalently binds to Cys668 within the EZH2-SET domain, enhancing EZH2 degradation and inhibiting tumor growth	[137]
		Astemizole	Diffuse large B-cell lymphoma cell lines	Disrupting the EZH2-EED interaction of polycomb repressive complex 2	[138]
DOT1L inhibitors		UNC0642 and EPZ-5676	Human U2OS Osteosarcoma, Human leukaemia cell lines MV4-11	Inhibits of H3R2me2a and H3K79me2, cooperation between DOT1L and CARM1, increase apoptosis and reduced cell proliferation	[139]

(continued)

Table 2 (continued)

Epigenetic mechanism	Altered expression	DRUG	Disease	Observation	References
		EPZ004777	Leukemia cells	Strong affinity for the SAM-binding pocket of the protein	[140]
		EPZ004777	Colorectal cell lines	Marked reduction of cell viability and tumorigenicity	[141]
		Pinometostat	MLL-fusion leukemia	Inhibited H3K79 methylation and MLL-fusion target gene expression	[142]
		EPZ5676	Gastric cancer	Regulating cyclins and H3K79 methylation	[143]
LSD inhibitors		Tranylcypromine derivatives	Acute myeloid leukemia cells	Myeloid differentiation and *Gfi1b* and *Irf8* upregulation	[144]
		Arborinine	Cervical cancer	Activated caspase-dependent apoptosis, suppressed cancer cell migration by downregulating expression of key regulators of epithelial-mesenchymal transition	[145]
		Higenamine	MLL-rearranged leukemia therapy	Increases expression of LSD1 substrates H3K4me1 and H3K4me2	[146]
		Kavalacotones	Prostate cancer	Inhibition of development and progression of tumor cells	[147]

Abbreviation ↓ downregulation; ↑ upregulation

epigenetic processes that occur during our life. Fraga et al. observed that monozygotic twins have similar DNA methylation and histone acetylation patterns; while older twins have significant differences in these epigenetic patterns meaning that the environmental conditions and diet influence their epigenetic profile [154]. Epigenetic changes have been linked also to exercise and Denham et al. described in their review how exercise can change the epigenetic profile of people and how it can prevent several diseases. They also showed that the epigenetic changes induced by exercise are reversible [155]. Diet is also a very important factor in diseases and it was demonstrated that poor nutrition in mothers during pregnancy can be linked to different diseases in humans and mice and most of the mechanisms involved are epigenetic [156–158]. Another epigenetic factor that has been shown to have an important role in the heritability of health and diseases is non-coding RNAs, where psychological stress and low protein diet affect the expression level of sperm non-coding RNAs, and these alterations are transmitted to offspring [159–162]. Studies showed that aging and obesity can also modify the sperm epigenome in humans [163, 164]. Alcohol is an important factor that can dysregulate epigenetic mechanisms by inhibiting the activity of methionin synthase (MTR), methionine adenosyl transferase (MAT) and DNA methyltransferases (DNMTs) [165]. Rossi et la observed that alcohol consumption is correlated to colorectal cancer development [166]. Heterocyclic amines are known for their genotoxic effect, but the mechanism through which they activate carcinogenesis still is not well understood. A study on rats observed that 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) tumors have a specific signature of dysregulated miRNAs including let-7 family, mir-21, mir-126, mir-29c, mir-145, and mir-215 [167]. Rodriguez-Miguel et al. observed that high corn-oil diet of rats can induce epigenetic changes that can be related to breast cancer progression [168].

Another important factor that can modulate epigenetic changes is chronic inflammation. One factor that can induce chronic inflammation is stress. It was observed that stress can induce chronic inflammation in patients with thyroid disease, and in some cases by epigenetic regulation can induce thyroid cancer [169]. He et al. observed that chronic inflammation of colon mucosa have different methylation patterns in genes involved in cancer development like PIK3CA, AKT, MAPK, Ras, Wnt or TGFb [170]. Chronic inflammation in cystic fibrosis or chronic obstructive pulmonary disease is related to epigenetic reprogramming of airways macrophages, which in turn favor tissue damaging and diseases progression [171]. Ahmad et al. observed that inflammation in COVID-19, lung cancer and other imflammatory lung diseases are regulated by different miRNAs and environmental induced inflammation is strictly regulated by epigenetic changes [172]. Also, oncogenic viruses have been shown to influence carcinogenesis through epigenetic modifications, including DNA methylation, chromatin remodeling histone modification, long noncoding RNA, microRNA, and circular RNA [173]. Rattan et al. discussed in their review the importance of gut microbiome and epigenetic changes in hepatocellular carcinoma [174].

5 Conclusions and Perspectives

Throughout an increasing amount of studies, epigenetics became a very intricate field in the overall understanding of cancer initiation and progress. The majority of epigenetic deregulations are the results of genetic mutations of certain genes that encode enzymes involved in the functioning of the epigenetic machinery. The final products of these mutations, being either solitarily enzymes, catalytic subunits from protein complexes, or noncoding RNAs, interconnections between them can regulate, through different specific mechanisms, the access of transcription factors to gene promoters, facilitating either expression or repression of particular target genes. On the other hand, this indirect regulation creates a "ladder of a multistep processes" and therefore gives the opportunity to additionally influence these "intermediate steps" before they reach the worse outcomes. Fortunately, in comparison with already established mutations that trigger altered functioning of genes that play roles in initiating events in the tumorigenesis cascade, epigenetic alterations can be reversible due to their increased plasticity and sensitivity to environmental factors.

Epigenetic changes, including DNA methylation, histone modification, and noncoding RNA expression, have also been reported in a wide range of solid tumors, emphasizing important alteration in cell proliferation, apoptosis, invasion, or metastasis. Epigenetics enables us to explore the potential mechanism underlying cancer phenotypes. Great effort has been devoted to understanding the role of these epigenetic alterations involved during development and cancer progression. Precise techniques should be developed and standardized for epigenetic evaluation in the genome or from a specific population of cells to hopefully a few or even a single cell.

An important role is related to the crosstalk between DNA methylation and histone modifications regulated by different nuclear factors. Pharmacological restoration of the epigenetic balance of gene expression is used in biomarker discovery and as a therapeutic target for human cancers.

Validation of novel epigenetics biomarkers will assist in diagnosis, prediction of drug response and eventually identifying the responsive patients. All in all, multidisciplinary field researchers need to work together to optimize the drug engineering process (novel compounds or drug derivatives from existing ones, in different combinations) to be tested in preclinical and clinical trials. The main aspiration is to translate epigenetic therapy into the clinic for the treatment of cancers and tailor effective strategies based on cancer types and epigenome-specific alterations. For this a better understanding of anticipatory processes in the living becomes a preliminary. We make reference here only to a suggestion originating from the biomolecular scientist Harry Rubin (communicated in Nadin, [175])—healthy cells keep cancer cells under control. Only when the anticipatory function is affected, does the cancerous cells get out of control. Louie [176] defined Nadin as the "anticipation guru"—enough for us to take his reference to cancer and anticipation at heart.

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Revisiting an Old Question

Genetic Anticipation: Fact or Artifact, Genetics or Epigenetics?

Arturas Petronis, James L. Kennedy, and Andrew D. Paterson

F. Clarke Fraser in his Aug 16 commentary [1] discusses potential biases in the study of genetic anticipation, and raises the interesting question of whether apparent differences in the age of disease onset between generations are a true biological occurrence or statistical artifact. Furthermore, he suggests the development of sophisticated statistical analysis to take into account the biases before beginning the search for unstable trinucleotide repeats, which are thought to underlie genetic anticipation. In fact, several new statistical approaches have been suggested for the study of intergenerational differences in the age of onset (reviewed in ref [2]). These approaches take into account the age difference between generations, but not such complex issues as fecundity biases, cohort effect, or assortative mating leading to bilineal transmission. Large, prospective, population-based datasets are rarely available, and if such studies were instigated now, the results would not be available in our lifetime.

The parallel use of molecular genetic strategies, however, might be immediately productive. On the basis of the unstable DNA diseases described so far, the presence of genetic anticipation infers unstable trinucleotide repeats, and various experimental approaches have been developed specifically to detect such expansions. Screening for trinucleotide sequences may help to address the role of trinucleotide repeats and anticipation in a specific disorder. However, this role is confounded by the possibility that other repeat expansions apart from trinucleotides might also be unstable and pathogenic, which would require a large number of candidate repetitive sequences to be tested.

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Recent findings from non-human sources [3, 4] and unstable DNA diseases [5] provide some evidence that epigenetic factors may also be involved in events similar to genetic anticipation.

Epigenetics consists of inherited and acquired factors that are not based on DNA sequence; they include DNA methylation and chromatin conformation. Epigenetic factors are involved in a wide range of genome functions including gene expression, genomic imprinting, developing, ageing, genetic recombination, DNA replication timing, DNA repair, and mutagenesis. Epigenetic mechanisms that might result in genetic anticipation are more likely to occur in complex diseases such as cancers, diabetes, or mental illness. It seems less likely that trinucleotide repeat expansions, which have been detected predominantly in simple Mendelian degenerative disorders, are the cause of genetic anticipation in complex diseases.

Despite the controversies surrounding genetic anticipation in human diseases, it is important to develop falsifiable hypotheses for clinical and molecular aspects of intergenerational comparisons of age at onset. The molecular mechanisms that trigger disease at a specific age have not been thoroughly investigated in human morbid genetics. Why age at onset is delayed remains unclear for common human diseases with a genetic predisposition, such as cancers or Alzheimer's disease, in which in some familial cases, mutant genes are present from birth. Studies of genetic anticipation could be the starting point for the clarification of molecular mechanisms responsible for the determination of age at onset of a disease.

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Epigenetics and Anticipatory Processes: From the Empirical to Foundational Aspects

Mihai Nadin

Abstract The making and remaking of the living can be described from a variety of perspectives. The genetic and epigenetic aspects of life dynamics are focused on the reproduction of organisms. Reproduction of life is never a repeat, but rather always an original. The anticipatory nature of life is ontological in nature. There is no life in the absence of anticipatory processes. Understanding interaction is the premise for a coherent foundation for the study of the relation between epigenetics and anticipation.

Keywords Interaction · Creativity · Non-determinism · Multicausality · Meaning

1 Conundrum

Epigenetics goes back to Aristotle:

> For e.g., an animal does not become at the same time an animal and a man or a horse or any other particular animal. For the end is developed last, and the peculiar character of the species is the end of the generation in each individual.

(Although not everyone agrees on the significance of his findings, [1, 2]).

This view distinguishes itself from the doctrine of preformation accepted during Aristotle's time. Instead of agreeing that the "end" features are fully formed in the zygote, the Stagirite argued in favor of gradual development from an undifferentiated origin, i.e., from the genesis. All this was based on empirical observations. He called the process *epigenesis* (Aristotle, *On the Generation of Animals*). In 1942, Conrad Waddington [3] focused on "the processes…by which the genes of the genotype bring about phenotypic effects." In defining the "epigenotype," Waddington echoes Aristotle's idea: "…between genotype and phenotype, and connecting them to each other, there lies a whole complex of developmental processes." His view, not unlike

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Aristotles', is based on the empirical. (We are not rehashing the history of the concept, but rather taking note of significant moments.)

Not surprisingly, *anticipation* goes back to Aristotle as well [4]

> .... if every instrument could accomplish its own work, obeying or anticipating the will of others ... if the shuttle weaved and the pick touched the lyre without a hand to guide them, chief workmen would not want servants, nor masters' slaves.

The notion of *prolepsis*, signifying foresight, originated at that time. *Antecapere ergo sum* is the formulation advanced [5] as the counterclaim to Descartes's "Dubito ergo sum"—more precisely, opposing anticipatory action to reaction (as a reductionist-deterministic process). It took almost as long as the time between Aristotle and Waddington's interest in epigenetics until Whitehead [6] suggested that each process involves the past and anticipation of future possibilities. Inspired by Whitehead and Burgers [7] went on to identify choice as coextensive with anticipation. Bennett [8] suggested that anticipation is "the basis for adaptation." After that, psychologists gladly adopted the subject, but missed its meaning. Before all of them, however, there was an impressive Russian/Soviet School—N. A. Bernstein, Alexei Ukhtomsky, Natalia Bekhtereva, Peter Anokhin, Dimitri Uznadze, Ivane Beritashvili, and Alexander Luria belonged to this group (whose work is still insufficiently acknowledged). Their activity was documented [9, 10]. Yet again: empirical evidence undergirded a rich production of breakthrough concepts waiting to be integrated into the body of knowledge of the science of life.

These preliminary notes on anticipation are also not intended to rehash history. Rather, the historic record serves as background for identifying a first conundrum: Given the significance of epigenetics and anticipation, how come the scientific community's acceptance of these processes was so slow? Moreover, how come the foundational work, in the absence of which knowledge is reduced to the descriptive, is avoided, even by those who currently seem to be attracted by phenomena epigenetic in nature or by anticipation-based activities? In our days, there are conferences: most recent is EpiSyStem: Stem Cell epigenetics (July 2022 Milan, Italy); and Anticipation 2022 (Tempe, Arizona USA), where even the chief of the Federation of the Huni Kui people will speak (in full tribal gear). There are journals, book series, endowed chairs, and everything else that reflects the search for an academic niche by using attractive keywords. There is no difference between such headlines as "Mother Knows Best" [11]; "Epigenetics: The Sins of the Father [12]; "Grandma's Experiences Leave a Mark on Your Genes" [13]; "Sperm epigenetics and influence of environmental factors" [14]; and the subject of various funding applications (submitted to the National Science Foundation/NSF, the National Institutes of Health/NIH, or to DARPA). The same holds true for subjects regarding anticipation, which the once illustrious discipline of Future Studies is trying to integrate (to the extent of renaming itself in order to get some legitimacy). "Anticipating a Breakdown" ([15], medically reviewed by White), "Hospicing Modernity" [16], "An Impending Breakup" [17], and so on belong to productions in which the word "anticipation" is used, but unfortunately in a manner that has nothing to do with what it actually means.

That anticipation processes are definitory of the living remains almost a tabu subject. The fundamental aspect of how the possible future becomes part of anticipation action is either ignored or sacrificed for the machinist view dominated by probabilistic inference from the past. Empirical evidence is replaced by convenient data processing of probabilistic phenomena. Thus, the vicious circle of proving a false premise by generalizing from outcomes conditioned by such a premise is closed. For the sake of explaining this situation, let us examine the nature of the knowledge to be gained if an appropriate foundation is established.

2 "Knowing That" and "Knowing How" Revisited

Rejecting the "official doctrine" of' Cartesian dualism, which ascertains that the mind and body are distinct, Ryle [18] distinguished between the "knowing that" and "knowing how." His famous example is riding a bike. You don't need to study anatomy, or physiology, or physics, never mind chemistry, in order to eventually discover how to place your feet on the pedals and steer the bicycle, and to keep going if you want to maintain balance. "Knowledge that" is not, at least for Ryle's example, a condition for "knowledge how." But the majority of those who use machines—such as cars, dishwashers, iPhones, etc.—have no idea (or even wrong ideas) of how they work, i.e., have no "knowledge that," or have the wrong explanations, even against evidence. More interesting yet: enamored of measuring everything, the majority of people relying on data (from measurements) have no idea how the data—the premises upon which machines function—are harvested. This is the epistemological "Achilles Heel" of our time. It is easy to notice that in its current state, "riding the bicycle" of epigenetics or of anticipation is dominated by the "knowing how": operational knowledge as a skill, in the absence of understanding the science upon which machines are conceived and measurements are carried out. Indeed, genetic sequencing—find the order in which the four nucleotides that make up a DNA strand are connected—is the bicycle. Given the enormous investment in "measuring" the DNA—the humungous genome project—it comes as no surprise that genetics is even defined in connection to it. The underlying genetic (genotype) of cell activity (resulting in the phenotype) is important, but the "bicycle of life" depends on more than the DNA, especially more than the model currently in use (Fig. 1).

The standard (by no means unanimously accepted) definition of epigenetics is the study of heritable changes of DNA, not involving changes in a DNA sequence, that regulate gene expression [20, 21]. In respect to anticipation, the standard definition (also by no means unanimously accepted) is: a system whose current state is determined by a predicted future state [22]. It is easy to see why neither epigenetics nor anticipation research went beyond the deterministic understanding of the dynamics of the living that corresponds to the Cartesian Revolution. DNA was declared, and is actively promoted, as the blueprint of life. In other words, based on this idea, everything that the living endowed with DNA features is the expression of elements making up the double-stranded helix famously discovered by Watson

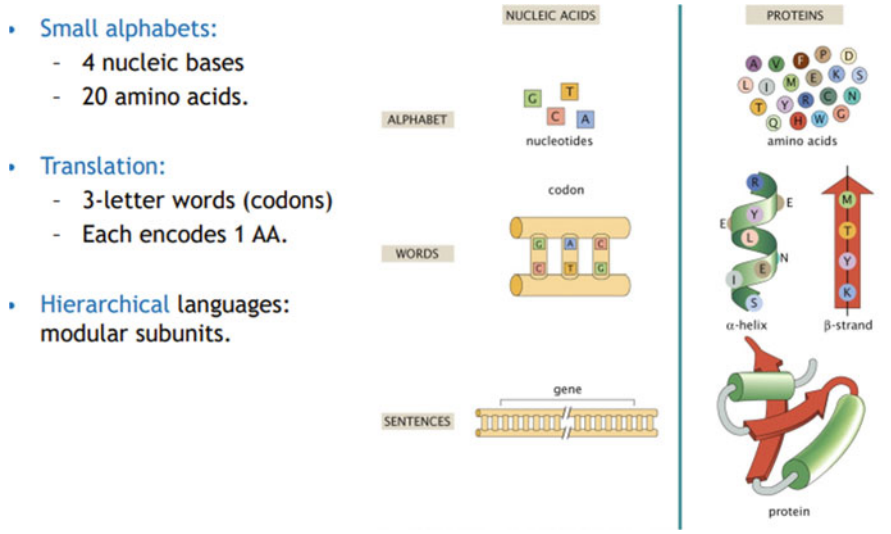

Fig. 1 Living matter theory according to Tsvi Tlusty (Lecture at the Physics Department at UNIST, [19])

and Crick. (Another individual, Rosalind Franklin, was also involved, but this is a different story.) Epigenetics is usually commissioned to prove that this is the case. Unfortunately, challenging this perspective has no place within the current genetic dogma.

A far as anticipatory processes are concerned, Rosen's exceptional contributions overshadow any ideas prior to his (his intellectual horizon was very broad, and he was aware of work done by others before his time). Moreover, those who follow in his footsteps ignore the contradiction implicit in the definition quoted above. Prediction—which his definition conjures—is antithetical to anticipation: to predict is to generalize from the cause-and-effect sequence, exactly what Rosen explicitly tried to avoid, or at least to suggest that it cannot explain anticipatory action.

The first conundrum—from low level of acceptance to forceful falsification of the epistemological premises—is not easy to overcome. Unless the scientific community takes note of the implicit limitations of faulty definitions, "knowing how" remains the only outcome, to the detriment of the ontological foundation expressed through "knowing that." It is therefore not surprising that science hostage to the Cartesian understanding of the dynamics of reality ceased to be productive, becoming a technological enterprise lacking in vision. The consequential nature of epigenetics, as well as of a science of anticipatory processes, was, so far, undermined by the confused epistemological grounding in reductionist-determinism. The learning cell is anticipatory; DNA is not. It is a stable chemical, with a double-stranded structure, incapable of learning. It is "a list of ingredients," but not a plan for action.

3 Darwin, Lamarck, and the Octopus

Darwin's *On the Origin of Species by Means of Natural Selection* [23], with its "detecting the smallest grain in the balance of fitness," projects a perspective of phenotype as the outcome of various traits: "The grain will determine which individual shall live and which shall die…." That Darwin was influenced by Jean-Baptiste de Lamarck is well known. Still, their views were deemed exclusive of each other, until Jablonka and Lamb [24] advanced the idea of a possible complementary view in the tile of their book, *Epigenetic Inheritance and Evolution. The Lamarckian Dimension.* This suggestion is relevant as we discuss Epigenetics and Anticipation because each of these knowledge domains ascertains complementarity: epigenetics to genetics, anticipation to reaction. Lamarckian inheritance and population genetics can be seen as reciprocally exclusive, or they can be seen in their unity.

To exemplify the thought, let us take a recent explanation of the self-destructive pattern of octopuses [25, 26]. The genome of the octopus comprises 33,000 protein-coding genes [27]—more than what humans have. Its evolutionary development ranges over 500 million years [29–31]. The octopus is a living creature with a large brain and an elaborate nervous system. From an evolutionary perspective, what attract attention are the eyes (which people who grew up in the age of the digital camera often describe as camera-like), the extremely flexible body, and very rapid change in color and shape. Under the scrutiny of geneticists, some [32] go as far as to question the possibility of applying to octopuses (of which there is quite a variety) localized Darwinian evolution on Earth. They advance the hypothesis of an extraterrestrial origin: "given our current knowledge of the biology of comets and their debris, the new genes and their viral drivers most likely came from space" [32, p. 12]. Be this as it may—an idea that will be either ignored or derided—it does not explain self-injuring and self-destruction in an organism often mentioned as "extremely intelligent" [33]. Empirical data (copiously shared by Wang et al. [25]), documents that post-insemination process, the male dies, while the females brood their eggs, starting what is usually called fasting, and undergoing physiological loss of function. There is what can be described as self-injury—people (scientists or not) who witnessed it are at a loss to describe this kind of behavior. Death appears not as the outcome of disease or injury (through predators), but rather as suicide—to use a term describing human behavior. First surprise: removal of the optical glands leads to a reverse path: the female octopus abandons her eggs and follows a normal path: feeding is resumed, even new mating takes place. The lifespan extension is ca. 40%, living longer than intact octopuses do.

No doubt, there is a lot to be learned about a self-destroying organism; three other aquatic animals also seem to be suicidal: salmon, dolphins, whales. However, a lot depends on the perspective of the inquiry [34]. Evidently, ending one's life is not characteristic of any physical or chemical process. There must be be life, which non-living matter does not have, in order for it to be terminated. Therefore, logically, to study the self-destruction of octopus life (or, for that matter, of dolphins, salmon,

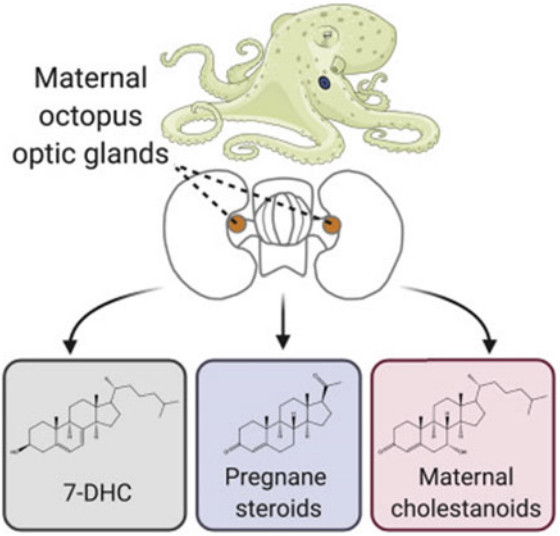

Fig. 2 This is the Graphical Abstract of the article Steroid hormones of the octopus self-destruct system, May 12, 2022, *Current Biology*. Reproduced with permission from Elsevier

starfish, and even a whale, of lemmings, bees, ants, or of human suicide; [35, 36]) has to be informed by

(a) accepting the difference between non-living matter and living matter;
(b) developing means and methods adequate to describing change (including end of life) in the living.

For the sake of argument, let us describe what was done in order to conclude that the explanation is "an imbalance between 7-DHC and cholesterol" [37] that "can dramatically alter steroidogenesis (Fig. 2).

All is based on transcriptomic findings, a molecular biology approach. Like all genetics-driven reductionism, you first kill your subject. Octopuses were bought, "animals were definitely sexed […] females with mature ovaries, ovarian follicles, and no evidence of fertilized eggs were positively identified as unmated females. Indeed, the EU Directive 2110/63EU Guidelines on cephalopod use were strictly observed. After anesthetizing the subjects, following perfusion "the animal was decerebrated [25]."

No need to reproduce further details. Although one question cannot be avoided: Why kill them instead of collecting a specimen that died naturally? What followed is the Sanger sequencing. Not different from any and all sequencing: you take the living, kill it, and then look for the chemistry—the sequence of nucleotides in the DNA—corresponding to life phenomena in order to explain them. Genetic reductionism is a form of chemistry reductionism: find the chemical elements to be associated with a life phenomenon. It is applied to bacteria sequencing, and to animal and plant sequencing. What we learned so far about the behavior of octopuses, or for that matter about plant dynamics, or the nature of Covid-19 infection is that a formidable

technology is available for producing extremely precise descriptions of the chemistry involved in life. But since there is no explanatory power in such descriptions; moreover, since what is measured might reflect the decerebration; mor than what leads to suicide, we face new questions. The conundrum of precision to the detriment of meaning becomes apparent. Data-rich and knowledge-poor is equivalent to riding Ryle's bicycle, driving your car, or piloting a private jet without understanding what they are and how they function. Worse yet: if you take them apart, there is no riding anymore. Sequencing describes in detail what they are made of but does not explain how they function. In the case of organisms, what is eliminated in the genetic sequencing is the definitory characteristic of life: its anticipatory nature. The living cell is anticipatory in its interactions with other cells (adjacent or remote); DNA is a stable chemical compound with a well-determined structure. There is no anticipatory process at the DNA level.

4 Blueprint of Life?

This is the juncture at which the legitimacy of epigenetics becomes evident. And also an instance of the unresolved conflict between those who reduce everything to the genome and those who realize that the complementary dimension defined as epigenetics is essential for understanding the dynamics of life. In particular, phenotype variability, empirically documented, raised a question impossible to ignore in view of the genetic explanation advanced so far. Does Darwin's original view, which accepted a Lamarckian model—the transmission of characteristics developed during life—explain the random germline mutations followed by natural selection on the progeny? Measuring mutation rates and mapping genotype-to-phenotype processes evinced not only the nondeterministic nature of such processes, but also the variation in their timescale [38]. Arguments used for defining the ability of organisms to adapt to changes in the environment suggest the need to define adaptive plasticity. Predator–prey cycles, climate changes (some cyclical, some not), immune system expression, and similar evince a component that belongs to the evolvable. Anticipatory processes are ahead of change. This can pertain to a short-time possible change (e.g., sexual expression before earthquakes or storms), or to long-term changes (such as geological events). There is empirical evidence for processes in which organisms "tune the timescale of their heritable variability to match the timescales of the acting selective pressure [38, p. 656]. Transgenerational epigenetics responses to environmental challenges [39] and premature attractiveness (in anticipation of non-deterministic process affecting sperm quality and other stress factors) confirm the suggestion that epigenetic interventions are often in anticipation of factors undermining the life of some organisms.

With all this in mind, we need to be aware of the fact that the dominant view is that phenotype change is mediated through changes in the DNA sequence triggered by epigenetic modifications. However, "In recent years, the belief that the genetic code is the sole basis for biological inheritance has been challenged by the discovery

of trans-generational epigenetic inheritance" [40]. Environmentally induced phenotypes (persisting for generations)—due to environmental factors—are not only in reaction to natural cues or stress, but often anticipatory [41]. Immune priming in vertebrates and invertebrates is an example.

But when all is said and done, the Medawar and Medawar [42] formulation stands out: "Genetics proposes, epigenetics disposes." To exemplify, let us reference yet another success: a gene mutation, occurring rarely (below five percent of all cancer patients), diminishes the success of chemotherapy. Instead of shrinking during treatment, the tumors of these patients grow. An epigenetic intervention through Dostarlimab (a new drug) changes the situation [43]. Regarding recent attempts at understanding genetics, in particular, the "Anticipatory effects…can evolve if environments are predictable across generation" [44], the role of DNA gives this provocative formulation an even broader meaning. First came the reaction to the monetizing of genome testing: "overstating the real nature of our DNA and believing that it is more important than it is" [45]. Determining destiny and identity through DNA sequencing is a representation ingrained in our culture because scientists overstated their case, and because "spit-into-the-tube" became fashionable, and profitable—even DNA from pets is now submitted to various commercial enterprises.

Even more relevant in discussing the consequences of doing biology under the guidance of the reductionist-deterministic flag is the realization, timid as it is, that "DNA may not be the blueprint for life—just as scrambled list of ingredients." This is the title of a press release from the University of Maryland. The peer-reviewed papers are from the *Journal of the Royal Society Interface* and in *BioEssays*. Inheritance, in Antony Jose's [47, 48] model, is seen as the outcome of a process involving entities (the genome, but also other molecules in the cell), sensors that make endogenous and exogenous interactions possible), and properties (such as arrangements of a molecule, concentration, proximity, etc.). One easily recognizes the inspiration: computer science, actually the machine view of the living—yet another conundrum to be aware of: To which extent is the real (biological process) and its model (computer, or DNA, or whatever) equivalent? Better yet: To what extent is inferring from the surrogate (no longer the monkey or the mouse or the rat, but the computer model) to the dynamics of life justified? The "ingredients of a cake", i.e., "the recipe coding for thousands of proteins that interact with each other and with the environment" does not mechanically reproduce in the "real cake," i.e., in the variety of organisms, none identical with each other. Machines make identical copies; the cell makes "different" copies. That is why neither von Neumann [48], with his model of "self-reproducing automata," nor those following in his footsteps (Antony Jose is one of them) succeed in defining life and how change takes place in the living.

Epigenetics offers a cognitive path towards resolving the conundrum of confusing the real and its representation. What it convincingly proved in its recent history—still under the tutelage of genetics—is that there are many ways to bring to life the chemistry underlying change in the living. Before suggesting our own view on the matter, as it pertains to anticipatory processes and to epigenetics, let us revisit—without any claim of exhausting the subject—how epigenetic interactions affect life.

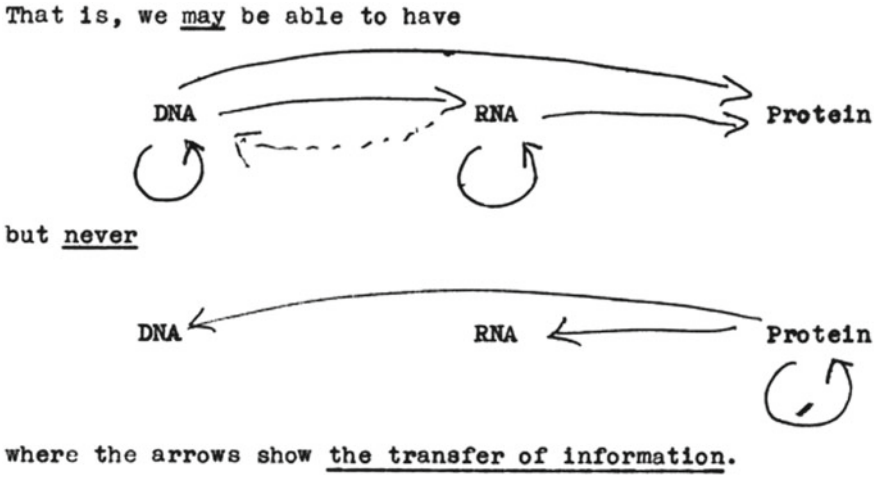

Fig. 3 Francis Crick's unpublished 1956 sketch of the central dogma (Image Wellcome Library, London)

The central dogma of biogenetics sees life as the expression of a sequence: DNA → RNA → protein (Fig. 3).

In a report on a meeting on "Frontiers in epigenetic chemical biology," Ganesan [49] takes note of the fact that "phenotype diversity of life on earth is mirrored by an equal diversity of hereditary processes at the molecular level." Even the mercurial (and often wrong) John D. Watson (co-discoverer of the DNA double helix) realized that "You can inherit something beyond the DNA sequence."

Empirical evidence shows that there are many processes that affect gene activity without changing the DNA sequence. So far, some of these seem to have stolen the limelight: methylation, acetylation, phosphorylation, ubiquitylation, sumolyation. DNA methylation (the focus of Szyf's contribution in this volume [76]) is the easiest to study with the available measurement technology (Fig. 4).

But there is also chromatic modification, and there are quite a number of other epigenetic paths. More important: in addition to matter (various substances) that can lead to changes in genetic expression, there is a rich body of accumulated evidence concerning licking, grooming, a variety of nursing paths, and many other behavioral influences. Although some are the result of observing surrogates (mice, rats, monkeys, etc.), there is enough reason to assume that humans behave similarly. And, of course, there are environmental factors, including interactions within species or among different species. Regulatory proteins, of instance, reflect nutrient availability [50]. Transcription patterns in bacteria are evidence that they anticipate environmental changes (storms, earthquakes). As cells grow and divide (the so-called cell cycle), they undergo interphases preliminary to mitosis. The new cell (daughter cell) will undergo different stages before the two copies of the genetic material (resulting from mitosis) enter into the dynamics of transgenerational epigenetic inheritance. The recent completion of the genome (annotation of the previously missing 8% of

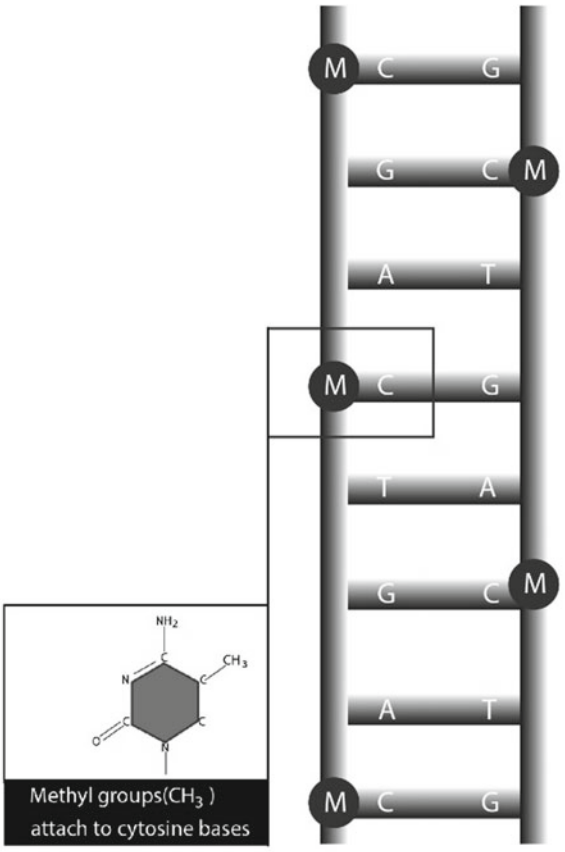

Fig. 4 DNA methylation regulates gene expression

it) provided new means for understanding epigenetic processes [51]. and made the community of researchers aware of the open questions they are trying to address. For instance, there are processes not yet identified or, even worse, attributed to factors that align with the reductionist-deterministic doctrine to the detriment of ignoring the nondeterministic nature of life processes [52, 53]. Given the various timing involved in genetic processes, it is probably justified to assume that the genome project will never be concluded since it is an open-ended evolving entity. To know the human is to understand its never-ending change—even though in the current view the DNA seems pretty stable. But who knows what two, three, four generations in the future will bring with them?

These preliminary considerations are the result of identifying conundrums waiting to be addressed. They are an argument in favor of providing an alternative path to understanding epigenetics and to connect it to anticipatory processes that constitute the necessary condition for change in the living. In short, foundational work, which the "know how" cannot provide, is not a luxury, but a necessity if we want to make "know that" the premise for actionable knowledge.

5 Change—The Anticipatory Condition of Epigenetic Processes

The National Library of Medicine (NLM) at the US National Institutes of Health (NIH) makes available on PubMed Central, a free full-text archive of biomedical and life sciences journal literature. Searching for keywords (single, or for more elaborate descriptions) affords an attractive meta-perspective. The titles (Who can read everything given that the curve of increasing publications is steep?) suggest that both *anticipation* and *epigenetics* are often associated with change. Weinhold [54] frames his examination of "a wide variety of illnesses, behaviors, and other health indicators" from the perspective of changes in gene functions by emphatically ascertaining "Epigenetics: The Science of Change." His list of health indicators is broad: "cancers of almost all types, cognitive dysfunction, and respiratory, cardiovascular, reproductive, autoimmune, and neurobehavioral illnesses." What follows is even more indicative of the almost open-ended kinds of processes involved:

> Known or suspected drivers behind epigenetic processes include many agents…heavy metals, pesticides, diesel exhaust, tobacco smoke, polycyclic aromatic hydrocarbons, hormones, radioactivity, viruses, bacteria, and basic nutrients.

From a cognitive perspective, it helps to distinguish between reactions (to substances, to stress of all kinds, to danger, etc.)—for which we have descriptions in the language of physics and chemistry—and anticipatory actions (such as the immune response, or any method of prevention such as a healthy diet and physical exercise,). What they have in common is that they are the expression of life. Since epigenetics and anticipatory processes share in the way they manifest themselves, what follows is an attempt to frame the subject within a conceptual foundation for a science of change. If successful, it could constitute a premise for advancing the agenda of a science that integrates reaction (deterministic in nature) and anticipation (non-deterministic in nature). Such a research perspective benefits from both the reductionist experiment (focused on the make-up of matter, atoms, molecules, genes, etc.) and the holistic (focused on the open-ended timeline of life processes, i.e., narration of life).

Axiom 1
All there is is the outcome of change.

Regardless of the viewpoint adopted regarding the beginnings of life on Earth (or in the Universe), it is clear that our very existence, as observers of reality (including our own), is the outcome of change. The most recent hypotheses are yet other attempts to transcend the "primordial soup" of life model (mix the right elements and provide an environment propitious to their combining). The claim is that it all started with the RNA–nucleoside triphosphates percolate through basaltic glass [55]. Whether this idea will withstand further examination or not, it aligns with the Axiom articulated above. Change is the origin of all there is.

In describing change, based on observations that can range between the casual, the experimental, or the empirical, what becomes apparent is that.

Lemma 1

Change can be necessary or contingent.

Just to build upon the RNA hypothesis regarding the first genetic material: the percolation in question is deterministic. Moreover, it can be only contingent since there is no necessity to its happening. The degree of the necessity of change and the nature of change (deterministic or non-deterministic) correspond to the fundamental condition of the matter in which the changing entity is embodied: living or non-living.

The WHY? of change regardless of its nature—i.e., including the emergence of life on Earth—is straightforward: interactions. The meaning of the word *interaction*: the way in which everything that exists influences each other, at all levels of their existence. This pertains to all that there is: lifeless matter (the non-living) and the living embodied in matter. Interaction and causality are of a different condition. Interactions between two entities or among several entities take place in in a never-ending back-and-forth of energy exchange. Within the deterministic model, causes are described through one-way arrows pointing to effects. They are also indicative of the order in the sequence: intervals between cause and effect are called time. This is, of course, a misnomer. Time is different from the interval between successive events. It is in fact more the rhythm in which change takes place—sometimes slower, sometimes faster. In the science grounded on measurements—always the same—intervals between measurements are also confounded with time. The consequence is evident: machines for counting intervals, such as the clock, effectively replace time. When Einstein described the space–time curving, his theory is not about intervals or distances. "What's the time?"—the usual question of the age when clocks were not as abundant as computers are today—actually meant "What interval was measured" in reference to an arbitrary beginning of the day, or the hour. This is inconsequential in respect to the non-living, where there is no birth and no death to reference to the arbitrary record of duration. However, it cannot be ignored in defining lived time—behind which age, disease, and death hide—as change of a nature different from that of the non-living. Interactions are variable in intensity and quality, as well as in their rhythm (some are faster, some are slower, some are continuous, some are granular). Causes can be sequential in nature: one, or many, after the other; or they can be configurational; or simultaneous.

The WHY? of interactions has its origin in the integrated nature of all that there is. In particular, matter and energy, which make up everything, are interlocked in the identity of all that there is, as well as in all that will be. For an observer, the relative morphological stability, i.e., the form, of things at all levels of their existence is the immediate consequence of the intertwining of matter and energy.[1] Of course, the relative stability of the form of a stone is fundamentally different from that of

[1] Physics developed the theory of forces (e.g., gravity, electromagnetism, strong, weak) in order to explain this.

a particular organism (whose form changes between conception, birth, and death), and from that of a species.

The complementarity of living matter and non-living matter is reflected in the attempt to describe through entropy the decay of non-living matter, in contra-distinction to acknowledging the diminished entropy of living matter. Without probing here in depth the neg-entropic aspect of living matter, we can provide the empirical evidence: organisms are the phylogenetic memory of the process through which simpler life forms continuously evolve. They create themselves through interactions that do not simply reproduce the previous simple form, but actually contribute to their remaking as "more" (different) than what their precursors were. The distinction (living/non-living) is different in kind from the complementarity of light as wave and particle—advanced and demonstrated within a quantum mechanics view. The view of the electron as particle and wave, or of genetics and epigenetics, or of reaction and anticipation only exemplifies Niels Bohr's concept of complementarity, advanced as a characteristic of all there is. The interlocking of energy and matter explains the stable shape of a rock (what holds all the elements in place in a particular manner); fluids taking the form of a vessel; and gases filling a room. Birds of a feather, or sheep of a flock, or zebras of the same stripe, blades of grass, fish in a school are examples that can be understood only within the evolutionary process that characterizes the dynamics of life. The question of whether qualia—ideas, emotions, feelings, and all that is associated with this label—can be identified as well through the interlocking of matter and energy could be addressed only on account of an understanding of the specific interactions that define the living. That they are outcomes of specific interactions is the consequence of the first axiom.

Axiom 2

Self-preservation of life is instantiated in its change.

As a self-organized system, the living maintains its own interlocking of matter—living (cells, for example, or neurons) and non-living matter (such as the chemicals of the DNA)—and energy through metabolism. Moreover, it maintains the integrity of its instantiation in a particular form of life (the individual animal, plant, insect, bacterium) through self-repair, for which metabolism delivers matter and energy. Robert Rosen [56] tried to capture the process as he focused on the question "What is life?" In the formalism of the (M,R) systems, Rosen demonstrated that metabolism and self-repair are closed to efficient cause, which means that they are triggered from inside the living.

Metabolism and self-repair are the particular expressions of biological matter and energy interlocked over a limited viability domain that defines life. This is self-preservation. The description of the process (i.e., Axiom 2) is the pendant to the laws of conservation of mater and energy. It aligns with the discovery of the dual nature of light and, for that matter, of the electron. And extends to genetics and epigenetics.

> The living, as a subset of all that there is—according to von Neumann, given its negentropic nature it has to be preponderant in the reality-- is self-preserving of its individuality, and of its condition of being alive. Experimental evidence confirming the empirical basis of this pronouncement continues to accumulate [57]

The pendant to the law of energy conservation is the expression of matter and energy interlocked (for instance, in metabolism and self-repair) over a limited viability domain that defines life. This thought is as significant as the dual nature of light or of the electron. It takes its particular form in the relation between the genome and epigenetic factors of all kinds. Evidently, evolving from simple to more elaborate forms, the living does not contain instructions for how to do that. It cannot be pre-programed, as machines can be. To assume that the DNA is a blueprint is to ignore the creative nature of life: reproduction at higher levels of self-organization and with increasing interaction capabilities. Outside the viability domain, the living becomes lifeless matter, while often hosting the life of other species. In death, its dynamics is reduced to that of the non-living in which all change processes are triggered from outside (the physical forces). The viability domain is that of life making and remaking itself (self-creativity) through interactions supported by metabolism and self-repair. The interlocked matter and energy, in which the living is dynamically expressed, is the unity between sameness (birds of a feather, etc.) and difference (change over time, e.g., aging). The living undergoes transformation processes through which life is continuously re-created. Although metabolism and self-repair originate from inside (the dynamics of the living is endogenous), they are subject to interactions with the outside (exogenous) world.

The non-living manifests itself in its immediateness: the here-and-now of cause-and-effect interactions change due to actions from outside. The living, on account of self-preservation, extends from the immediate to the subsequent. This is where duration, as a particular expression of time, but not to be confused with it, emerges. Interactions characteristic of the living are several orders of magnitude more diverse, and of higher impact, than those defining the change of lifeless matter. Properties of lifeless matter are defined from the elementary particles making up the matter and energy processes involved in maintaining such properties. This is a bottom-up process—interactions (endogenous) among fermions, quarks, leptons, bosons, etc. to atoms and molecules, to physical entities (such as elements and things made from elements). Interactions with the world (exogenous)—some linear in nature, others non-linear—affect their properties, as well. The particular matter-energy interlock changes under their action (metals oxidates, stones crack, water acidifies, etc.). The description of their motion (trajectory, speed, continuity, etc.) is one possible manner, chosen by physics, to characterize change (relative position to each other). Descriptions of motion—such as those facilitated by the mathematical language of analysis—are actually an incomplete record of their change: the stone wearing down into sand, for instance, without changing its position in space; or, to recall the hypothesis of the RNA as the beginning of life, nucleoside triphosphates percolating through basalt glass (Fig. 5).

Properties of living matter result from complementary bottom-up—from the material make-up (electrons, atoms, molecules)—and top-down processes—from the cell down to its various components, from the brain to the genes. Even though the living is closed to efficient cause—that is, it changes due to its own dynamics—outside forces affect it as well, since life is embodied in matter (some alive, such as cells and neurons, some not living, such as the acids making up the DNA). Energy—endlessly

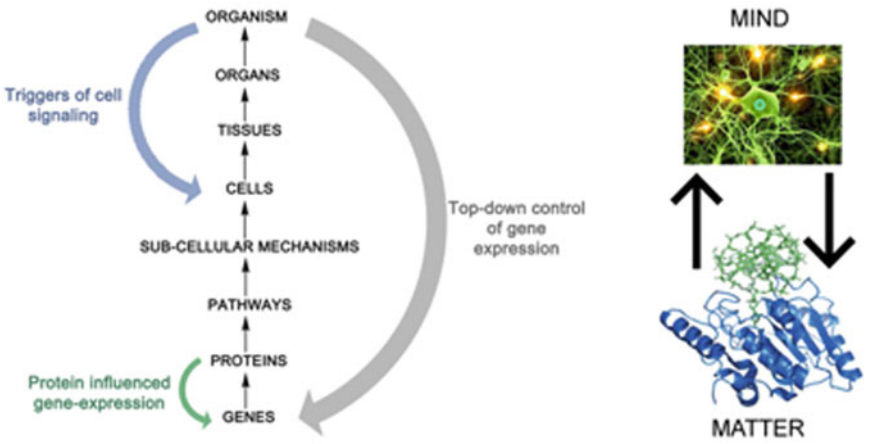

Fig. 5 Ellis [58] discussing top-down causation (cf. Interface Focus 2011)

transformed in intractable processes, but never created—is at work in affecting how matter supports (or sometimes undermines) life within the viability domain. Descriptions of motion, of things in the environment or of entities at the micro- or macro-scale (the domain of astrophysics), are relevant to physical entities. They are, however, of secondary significance in describing change in life: plants, for instance, don't literally move, although they can change their position. Ontogeny and phylogeny, as biological processes reflecting the dynamics of energy-matter interlocking, constitute specific behavioral patterns, as much as they define through, and are defined by, the material and energy make-up, in continuous renewal.

The never-ending change of any and all living entities—from insemination to birth to death—entails *creative processes*. Reproduction (sexual or asexual) is, from among a large variety of creative processes, the most prominent. To create is to make the past (what was, the genetics) and the present (what is) become a possible future (what might be). For this, perception of the future, i.e., a "sense" of what might happen, informed by, but not reducible to, sensorial perception and the rich cognitive activity this triggers, is a condition *sine qua non*. The human DNA is by some order of magnitude (25%-35%) less than that of some flowers. The immediate explanation: The "sense" of the subsequent—less defined for flowers than for humans or vertebrates—from which future is made up, is *anticipation*. Epigenetic interactions are often anticipatory. Anticipatory action orchestrates biological expression (such as motoric expression or neuronal activity) consonant with life self-preservation. The action, guided by anticipation—to which learning contributes meaning—transcends the action-reaction mode of lifeless matter—where meaning does not exist. In anticipatory action, *what is* becomes something that never existed before. Therefore, it can be qualified as creative. Flowers are "more" the same ("more" being a fuzzy qualifier) than humans are. In contradistinction to change in lifeless matter, which is essentially deterministic, anticipation-driven change is non-deterministic. It can

be successful—creativity as self-preservation ("art of surviving")—or not. The self-destruct behavior pattern of octopuses (an example we dwelled upon) invites an effort to understand the drive to live and give birth, and the realization of life cycles: beginnings and ends. No awareness is involved, rather, the interlocking of life-preserving factors. Some scientists speculate that the self-destruct action—many other species are known for similar patterns—is purposeful: to provide offspring with what they need to make it, or to protect others (aging termites protecting the "community," [59]).

Lemma 2

Anticipation processes underlie evolution.

The WHY? of evolution cannot be answered without understanding that it is grounded in anticipation. From the initial life forms on record to the current stage of life on earth, the vector of change is from the simple (distinct from the non-living in which it resides) to rather elaborate (changing itself and the world in which it acts). Self-preservation guides variation and selection, from the cellular level to that of species. It succeeds to the extent to which anticipatory processes lead to successful action. The WHY? of anticipation is straightforward: there is evolutionary change since anticipatory processes (as choices made) guide interactions beneficial to self-preservation of life. Being by nature non-deterministic, such processes do not prevent species extinction.

Lemma 3

There is anticipation because there is learning.

First a negative proof: If life were genetically predetermined ("programmed"), as reductionist-determinists ascertain, there would be no need for learning. Those who maintain that the DNA is the blueprint of life might argue that the living is "programmed" to learn. This would imply a teleological dimension: learning as final cause. And it would suggest that the medium—a non-living entity made of four chemical bases structured in a sequence arranged in two long strands making up a double helix—is the message. The wrong metaphor of genetic language leads to contradictions. Learning and protein production correspond to unrelated aspects of life: learning is necessary; protein creation, in which folding, a non-deterministic process is essential, is contingent. There is no lie without protein, but the folding is not predefined. The building blocks of proteins—the 20 amino acids specified by the three bases of the DNA (codons)—correspond to the syntax of life. Learning is pragmatic in nature, at a level where communication (inside and outside the organism) is established with the purpose of maintaining and perfecting life. There is no change at the genetic level; the DNA is what it is: elements in a fixed configuration. Learning, which is an epigenetic intervention, brings life into the DNA. The dynamics of life is the outcome of learning interactions.

Change in living matter is experiential. It leaves traces that eventually form knowledge—no matter how limited—of self and of the world in which the living unfolds. The WHY? of learning is subsumed in that of anticipation—ahead of the possible, of the contingent.

Anticipatory action takes place through biophysical and biochemical processes. Such processes are not reducible to the physical and chemical processes characteristic of lifeless matter. Lifeless matter and living matter are made of elements. However, molecules of life (proteins, carbohydrates, lipids, nucleic acids) are 96% composed of only six elements (carbon, hydrogen, oxygen, nitrogen, phosphorus, sulphur). The nature of interactions that each makes possible, moreover necessary, in order to ensure self-preservation of life, defines them as different from non-living molecules. Anticipation has an existential condition (cf. the WHY? of anticipation); that is, it is ontologically defined, not epistemologically constructed. Life self-preservation is accomplished through anticipatory processes bridging the now with the immediate or remote subsequent, i.e., the possible future. The same can be said about epigenetic processes. Epi-genesis is not a construct used to explain genetic expression, but rather a very rich ever-expanding set of interactions affecting gene expression (sometimes beneficial to life, other times detrimental—cf. the octopus self-destruct behavior discussed previously).

6 The Observables of Epigenetic Expression and Anticipatory Action

The understanding of change—the epistemology—conjures constructs such as time and space. However, as we shall find out, time and space pertinent to change in non-living matter is different in nature from the time and space of life. Within a unified systems perspective, the focus is on evaluation of observables over states of the system. As Einstein remarked, and as science shows, those who define the observables control the theory. This is evident when we compare the observables in Galileo's mechanics, in Newton 's physics, in relativity theory, and in quantum mechanics None of these apply to life. They describe the reality of a non-living universe. By extension, when von Neumann [48] submitted the model of self-reproducing automata, he took the cue from the self-reproducing living. Discarding the unrepeatability of life processes, he conceived of a mathematics that affords self-making—applied in our days to robots and other kinds of machines. His observables correspond to Turing machines, and thus contribute to making a mathematical construct the prototype of the digital algorithmic computation of those days. Not unexpectedly, the "chemists of life"—biochemistry—took a ride on the same bandwagon. They produced an explanation of self-reproduction focused on the DNA, or, by extension, on genetics. In every situation, observables are the outcome of simplification: a reduction. Since the complexity of life evades full and consistent descriptions (i.e., G-complexity [60]), reductionists explain life from a particular perspective (Fig. 6).

In the non-living, mapping from states to numbers captures the nature of change. Indeed, this change (the entropy of matter) is quantitative in nature. In the living, the mapping to numbers incompletely describes the nature of change, especially in view of the fact that the observables (making up the phase space) continuously change

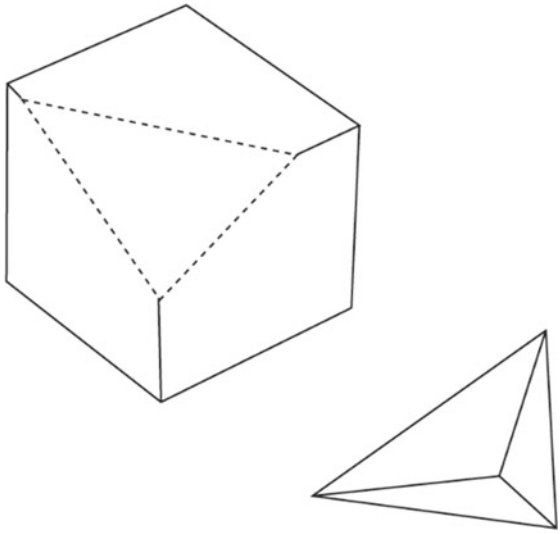

Fig. 6 The epistemological cut—selecting a limited aspect that can be fully and consistently described

[52]. To better account for the change in the living, it becomes necessary to perform mappings from states to meaning—their significance for the living process—as it applies to the self-preservation of life. Epigenetic interventions of material nature (e.g., use of drugs, such as in the treatment of rectal cancer, as mentioned above; [43] or any other form of interaction (spiritual influence, ecological factors, etc.) can be associated with genetic processes but are not reducible to them. They take place over time. Therefore, to understand them, sequences of maps must be generated, that is, a film sequence of the process subject to inquiry from the perspective of how each step (in the time series) is significant for the self-preservation of life. It is not enough to identify one or another process (methylation or chromatin modification) in the absence of the larger context. Just as an example: imprinting.[2] If one of the two alleles of a gene pair is "silenced" due to an epigenetic process, the allele expressed might be vulnerable (to microbes, or some toxin). Genes that can be imprinted are subject identification given the vulnerabilities associated with the process (Fig. 7).

In the reactive system of the non-living interactions, the state of the system depends on its past:

$$x(t) = f(x(t-1)). \tag{1}$$

Therefore, the description of the dynamics of lifeless matter is straightforward: its change is fully described through the variables relating the past to the present (characterized as duration and proximity). The number and variety of parameters describing the non-living is finite (even though it can be very large). Interactions in

[2] Imprinting: a rapid learning process that takes place early in the life of a social animal (such as a goose) and establishes a behavior pattern (such as recognition of and attraction to its own kind or a substitute). (Merriam-Webster).

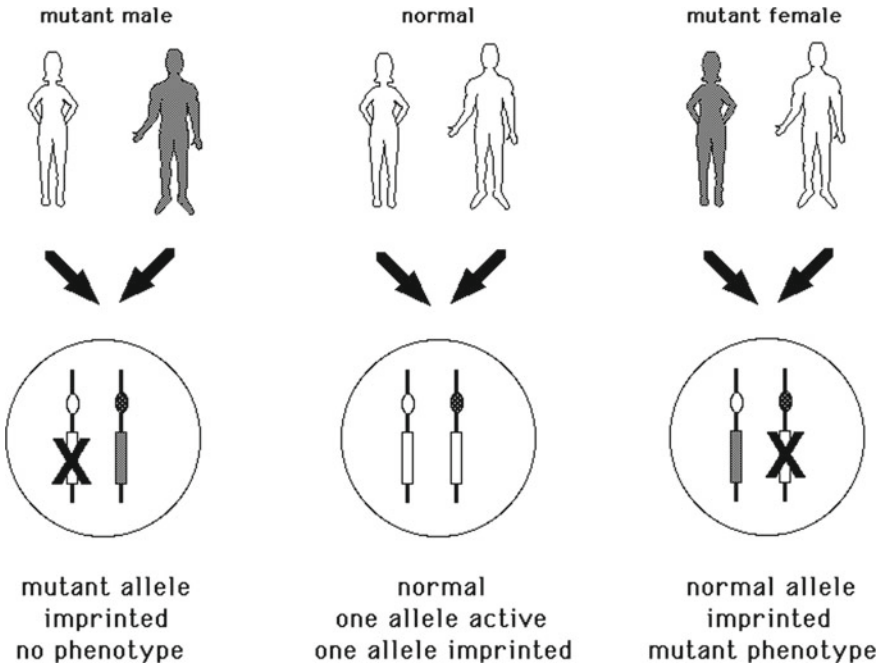

Fig. 7 Genes imprinted

lifeless matter and among non-living entities is described in the dynamics of action-reaction, i.e., deterministic causality (including, for instance, processes described in chaos theory, the mathematics of dynamic systems). Inferences from parts to the whole are possible because interactions through which matter and energy are interlocked are preserved (up to a certain scale). Variations (an expression of our imperfect descriptions) appear to average out. As part of the organism, the non-living, such as the DNA and genetic processes associated with it, can be measured—that is what sequencing, the dominant measuring process of our time, does. The deterministic machine called *compute*r provides the high analytic performance expected once the data reach a very high scale.

Living systems are anticipatory. The current state of an anticipatory system depends on past, current, and possible future states:

$$\mathrm{x(t)} = f(\mathrm{x(t-1)}, \mathrm{x(t)}, \mathrm{x(t+1)}) \tag{2}$$

The dynamics of the living cannot be described and explained without considering the possible future. DNA is blind to the future. It encapsulates past and, at most, might undergo accidents. The number of variables describing the dynamics of the living is as open-ended as the possible future-based choices it faces as it unfolds over its viability interval. The interlocking of energy and matter in the living makes

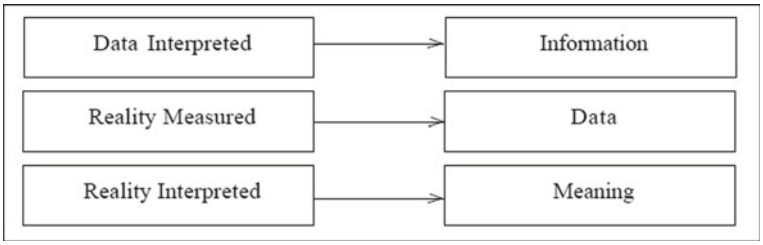

Fig. 8 Data: matter/meaning: life

possible the simultaneous condition of sameness (in species, in offspring) and difference (expressed as individuality, of which lifeless matter has none). Inferences from parts to the whole in the living are at best misleading. Interactions through which living matter and energy are interlocked is specific to each and every life level: cells, membranes, tissues, organisms, etc. Lifeless matter is homogenous—atoms, molecules, chemical elements, are each of the same nature. All electrons are the same. The elements have a specific composition that defines their identity (e.g., oxygen or hydrogen, copper or uranium). Life embodied in matter is heterogenous from the cell level to tissues, to organs, up to the organism. The identical is an identifier absent from anything living.

Lifeless matter neither reproduces nor replicates itself. Life self-preserves itself through replication, involving genetic elements (such as DNA molecules), but not limited to their chemistry. Reproduction is actually No-reproduction, but diversification. Intertwined sameness (of species) and difference (of individual organisms) correspond to creative change: life is always made from life, continued in never-repeated forms. Paradoxically: the pattern of no-pattern (Fig. 8).

Time and space, in the living are not a given stage on which things happen. Rather, they are the outcome of change, coextensive to change.

- Lifeless matter is describable through measurement/quantity, number, math—subject to falsification
- Life is describable through meaningful time series (narrations)—ambiguous.

To know (as in riding a bicycle, or carrying out genetic sequencing) is to experience HOW? *To know that* (i.e., what makes the action possible) is to understand WHY? [18]. In this respect, the study of the octopus's self-destruct behavior is perfectly justified. But the results depend on the perspective, i.e., the measurement means and methods. The killing of the organism as a preliminary to finding out why the living specimen behaves in some peculiar (to humans) manner diminishes the choice of observables. Of course, genetic sequencing will output what genetics is about: chemistry. But the behavior in question is different in nature from chemical reactions. It corresponds to interactions with other species (availability of sustenance), as well as with the environment. Does the octopus mother, without any explicit awareness of the possible future, sacrifice herself for the sake of the offspring? The notion of sacrifice corresponds to an anthropomorphic perspective: explain what is done in

Nature by assuming that it behaves like humans do. But the answer afforded via chemistry is also anthropomorphic: it must be steroidogenesis. Actually, genetic sequencing, unveiling the syntax of genetic processing, could not address questions pertinent to "know that."

Axiom 3
"Knowing that" is not experiential.

To know that (for instance, how epigenetic processes affect DNA expression) and to account for how "knowing that" changes the knowing subject is at the core of Popper's criterion of falsifiability [61]. Most knowledge in the living is implicit. It is expressed in the change experienced and results in changed patterns of behavior, i.e., in new forms of interaction. The WHY? question is irrelevant for the experienced. You bike without ever contemplating the WHY? question. For that matter, we live without knowing what life is. The human being observing change in nature is inclined to attribute a human dimension (anthropomorphizing, as explained above) to such change. The extreme reaction to this epistemological trap (we see ourselves in what we observe) is the attempt to create a context in which measurement replaces impression. In time, quite a number of means and methods for measuring have been conceived. The history of science documents such advances. What if it fails to do is what some scientists, fully dedicated to the knowledge domain they are active in, eventually realize. In his Nobel Prize acceptance speech, Albert Szent-György (Laureate in Physiology or Medicine, 1937) provides a good illustration of the thought:

> As scientists attempt to understand a living system, they move down from dimension to dimension, from one level of complexity to the next lower level. I followed this course in my own studies. I went from anatomy to the study of tissues, then to electron microscopy and chemistry, and finally to quantum mechanics. This downward journey through the scale of dimensions has its irony, for in my search for the secret of life, I ended up with atoms and electrons, which have no life at all. Somewhere along the line life has run out through my fingers. So, in my old age, I am now retracing my steps, trying to fight my way back.

This is extremely relevant in the context in which the observables—what we measure—change.

Let us recall examples from behavioral epigenetics. How change affects experience is reflected in the changed behavior. Behavioral epigenetics is illustrated by examples ranging from the individuals who were prenatally exposed to famine during the Dutch Hunger Winter to the offspring of Holocaust survivors. To understand change implies awareness of consequences: the children of the Dutch Hunger Winter of 1944–45 had, six decades later, less DNA methylation of the imprinted *IGF2* gene compared with their unexposed, same-sex siblings [62]. Of course, what is observed, i.e., measured, is different from what actually took place. Not surprisingly, there are scientists captive to measurement who dispute the findings related to the Dutch Hunger Winter or to the Holocaust survivors because genetic inferences taken out of the context of life are ambiguous by necessity. Beyond controversy is the need to understand living processes in a holistic context. Learning, as the multitude of

processes through which holistic anticipatory processes are informed, is expressed in accumulated understandings that pre-empt undesired experiences.

Being the axiom of life, self-preservation becomes by necessity the criterion for qualifying changes pertinent to the living: undesirable, creative, inconsequential. To know something has the immediateness of experiencing it and the subsequent action it informs.

To understand is by necessity an activity involving the change under inquiry, the inquiring subject, and all mediating entities between the two. To know how the change of lifeless matter affects the self-preservation of life is to form a representation of the possible interactions between them.

7 The Threshold of Complexity

The above-formulated axioms are premised on rich empirical evidence, as well as on experimental outcomes, including negative results (respectively, failed anticipation and epigenetic expression to the detriment of life) and failed replicability (discussed in [52]). What follows is an attempt to elaborate on the pronouncements within a method co-substantive with the subject. David Deutsch (*The Beginning of Infinity*, 2011) correctly described succeeding theories: Galileo's *Dialogue Concerning the Two Chief World Systems*, 1632; Newton's *Philosophiæ Naturalis Principia Mathematica*, 1687; Einstein's *On a Heuristic Viewpoint Concerning the Production and Transformation of Light*, 1905; and quantum mechanics. Of course, the correspondence principle holds: Galileo's mechanics is right—i.e., can be used and tested—until the moving objects are characterized by their mass, and therefore their interaction cannot be ignored; Newtons' mechanics (describing particular gravity-based interactions of non-living bodies) is right until the speed of movement comes close to that of light; Einstein's physics is right until Heisenberg's uncertainty principle (i.e., the quantum mechanics view) comes into play.

But each new paradigm—a breakthrough at the time it was articulated—ascertains discontinuity also: the mechanics of falling bodies (Galileo) is of local significance. Newton's view according to which the universe obeys the same laws of Nature introduces gravity as a force exerted upon interacting bodies; in Einstein's universe, there is no place for such a force: Earth's mass causes space–time to curve. In this distorted space–time, the shortest path (the geodesic) is no longer on a flat surface (plane), but on a sphere. Einstein's view on the limited speed of light is, in turn, challenged by the instantaneous entanglement of photons (which led him to write about "spukhafte Fernwirkung"—spooky action at a distance).

It is quite possible that anticipation, as definitory of the living, will prove to be a breakthrough, after centuries in which biological subjects have been explored from the perspective of physics and chemistry. The correspondence principle will have to be rewritten: the biological, above the threshold of complexity at which decidability is expected, seems to ascertain a view in which the physical is the particular case. Indeed, within the reductionist-deterministic premise of explaining

the world, the living has been a particular case, a subset of physics, or of the physio-chemical model of reality. The life sciences have operated under this assumption, and consequently, biology was corralled into biophysics and biochemistry. Given that life is non-decidable (for arguments, see [60])—i.e., as opposed to the non-living, it cannot be fully and consistently described—it follows that below the threshold of life, causality is by many orders of magnitude below that characteristic of life processes. Consider only the fact that genetics expression, focused on DNA, with its large data description, is much simpler than epigenetic interaction, and you have a vivid image of what the particular case is, and what the encompassing nature of life is. After all, the living can produce non-living entities; the inverse does not hold.

The most important consequence of this epistemological understanding is that change—and its causes—is key to efficiently distinguishing between biology and physics or chemistry. At this juncture, it becomes clear that science has reached, through the proper understanding of the living, a level of generality impossible within the focus on particles, atoms, molecules, etc., or chemical components such as DNA. Therefore, one cannot continue promoting the language of Descartes—who built upon Plato's "nothing can come without a cause" (*Timaeus*)—in addressing something that Descartes' axiom excluded: a cause that lies in the possible future. Einstein's message—"No problem can be solved from the same consciousness that created it. We must learn to see the world anew."—is in this sense more current than ever and pertains to anticipatory processes as well as to epigenetics. It makes little sense to couch anticipation within conceptions anchored in the past—or to legitimize it, in a castrated rendition within which the future is the outcome of probabilistic evaluation, within modes of arguments contrary to its condition. Once again: the same holds true for epigenetics, especially for couching it in genetics, and its surrender to the measurement technology associated with the genome.

8 Distinctions

To learn about the world is to learn about its change. Explanations of change within the physics-dominated understanding of the world or within the chemistry of genetics characterize only a small part of the dynamics of life. They return partial descriptions of non-living matter, leaving out what characterizes life. The very idea that change is of essence goes back to Heraclitus, who maintained that fire was the cause of change. If fire is understood as energy, we are not far from what science ascertains in our days. This idea is not contradicted within any conception of lifeless matter (physics, chemistry). Its relevance becomes clear when examining living matter, i.e., organisms. Anticipatory processes, in particular in the form of epigenetically triggered genetic functions, underlie change under the axiom of self-preservation of life. Obviously, as living observers learn about how things (living or not) change, they are subject to change as well. The circular nature of knowledge acquisition is significant because even in the conversation on the nature of who we are and what defines us, epigenetic influence is exercised. There is a continuous feedback cycle, resulting in

phenomena ranging from self-delusion (superstition and mysticism are examples) to self-motivation. Let us recall testimonies regarding a patient's will to live and how it affects the outcome of medical care. Such examples are not reproducible because they testify to the uniqueness of each person. But that would be a subject in itself. Change takes place on account of interactions among all that there is. Associated with this fundamental premise is the axiom of existence. All that there is—material or of a different nature (such as emotions, thoughts, cognitive constructs, etc.—is the outcome of change and becomes the locus of future change. In even simpler terms: regardless of the views one holds about the beginning of the universe, not to say the beginning of life or of humanity, even beginnings are the outcome of change leading to subsequent changes.

There is no place in this view for anything that would qualify as nothingness because interaction implies distinctions. Change multiplies distinctions. If it leveled them, it would outcome nothingness corresponding to absence not only of matter, but also of energy. From all the knowledge acquired so far, energy is subject to transformation, but not to exhaustion (into nothingness), and even less to self-generation.

It does not take sophisticated experiments to find out that change of lifeless matter and change of the living afford different perceptions under observation. A stone changes over time, as weather changes, or as it interacts with the living: seeds finding a niche in the smallest crack, all kinds of life forms seeking refuge near it, the chemical reactions between its constitutive elements and acids (in rain, urine, feces, etc.). All these can be measured, and are measured more and more, since measuring methods and measuring devices are continuously developed for this purpose. The data acquired represent various aspects of the change. The assumption of a complete description corresponds to the nature of the described (i.e., the stone in this case). To observe a newborn (a hatchling from an egg, a faun from a doe's womb, a plant from a seed) could also inspire measurement. Books were written that detail the apparatus for measuring what an egg is, what it is made of, how fecundation affects it, etc. Let's recall Aristotle's contribution to science demonstrated by his classic empirical observation of the growth of a chick inside an egg. There is no data, there is a record of change ("the film," the narration). The fanatics of measuring still seem unaware that numbers do not provide access to the creative dimension of change, i.e., how something that never existed comes into existence. Embryonic stem cells in interaction with fibroblast growth factors (FGFs) are primed to become a goldfinch, but not a copy of any existing one, rather a unique bird never yet encountered. This is where the anticipatory nature of epigenetic processes becomes evident. As already pointed out, the assumption of a complete description, under which genetics operates, is not realistic since the number of observables involved in the process changes as well.

We shall see what it takes to understand the difference between change in lifeless matter and in the living as we advance in defining the perspective from which such an understanding becomes possible. Let's take note of the fact that in this world of inexhaustible change, the understanding of the observers themselves, of who they are and how they take in the world to which we belong has changed over time.

Let's take one example: A large variety of eyes—on fish, butterflies, owls, octopuses, etc.—testifies to ways in which the living learned to see the world, and thus overcame the limits of only reacting to it. (Of course, the other senses were involved as well.) Dated in the Cambrian period, during which evolution seems to have known an accentuated dynamic, the eyes affected adaptations, and were affected by them. What today we call visual acuity, sensitivity, motion resolution, and color distinction were and are shaped by the environments in which organisms live. This is the answer to the WHY? Of such characteristics of vision. None other than Darwin suggested a progression from "an optic nerve" to what eventually became, in vertebrate evolution, a patch of photo receptors [63]. Empirical evidence, of the nature of Aristotle's observations of egg germination, suggests that evolution in itself is beneficially influenced by higher light sensitivity. Molecular biology made possible the retracing of the co-option of a protein from some other function to the formation of photosensitive cells. Genetic mechanisms were identified [64] in respect to the location of eyes in organisms as diverse as octopuses, mice, and fruit flies.

Most significant from the perspective pursued here is the fact that interactions with the world, enabled by sensory organs, are from early on not reduced to reaction. Light, of course, would lead to a response (defined in the context of interaction); this corresponds to the cause-and-effect physics of reaction. But seeking light, or for that matter, darkness (e.g., in order to avoid danger, to find a moist area where nourishment might be available) is anticipatory. This exemplifies a concrete path of life self-preservation dynamics. The light-sensitive protein opsin and the molecule facilitating color distinction make up the photo receptor cell (eyespot). Organisms of different species and types do not see the same image of an object within an environment. They distinguish *Umgebung* (the universe in which they live) in the self-preservation environment—*Umwelt*, as von Uexküll [65] called it. In some cases, the sensorial representation is transmitted to the brain (when e.g., sustaining circadian rhythm); in others, the sensorial guides action (reaction or anticipatory action). Cladonema (a sort of jellyfish) has no brain; the eyes seem to control the motoric directly. Molecular biology helps in understanding the intricate nature of what we take for granted when referring to the sensorial.

These minimal notes (from an extremely rich body of knowledge regarding vision) explain why Avicenna (eleventh century) thought that the eye is like a mirror—what is seen is a reflection on a mirror—while Plato (and some of his followers) hypothesized a spotlight view: the eyes put light on the things in the world. Aristotle, in opposition, described a receiving eye. It took some time until dissection would inform more advanced descriptions (of the retina, cornea iris, etc.) based upon which Galen arrived at an analogy with the lens, and to the binocular vision model. Changes in the understanding of what eyes are and in realizing how interactions facilitated by vision take place are amply documented. From early mytho-magical testimony to the Renaissance and up to Descartes (who understood neither vision nor the connection to cognitive processes), visual interaction facilitated by seeing is explained in a sequence that runs the gamut from the intuitive (based on immediate experiences) to the scientific. The lens, ascertained also through the instruments of the time,

succeeded as the most accepted description of the "hardware" of seeing; while evolution researchers still wonder why in some organisms the nerves are placed before the lens, and in others, behind. Rich data from a variety of experiments show that the epigenetics of taking the world in through vision is more complicated. What genetic methods usually leave aside–while trying to get to the reductionist end (name the gene of sight, of hearing, of smelling, etc.) or describe how the energy of sensory perception becomes a representation—is the holistic nature of perception. Anticipatory quality is achieved on account of the subtle integration of various stimuli. The senses interact: animals see many things on account of hearing them, smelling them, of touching them, etc. For the human, creating an image of what is anticipated—sometimes right, sometimes wrong—is part of the process. Anticipatory processes are non-deterministic. A deterministic conception, such as genetic reductionism or computational biology, does not make this understanding possible. Epigenetics, properly understood, helps in making clear that living processes are different in nature from mechanical processes.

Sensing, in its most limited sense (no pun intended) emerges as the types of interaction among incipient forms of life and between the and the environment, diversify and increase in intensity. Initially, sensing is probably of the nature of tactility: physical contact. (Regarding the evolutionary origin of sensory processing, see [66]) The notion of syncretism seems to more adequately capture the continuum of the spectrum of living interaction. Millions of years later (the Cambrian mentioned above) extended to smell, sight, hearing, etc. In the examples above the focus on seeing and the eye is meant to suggest the role of the eyes (whenever some are formed) in the change through which incipient life (no eyes as such, rather photosensitivity), of limited sensory abilities, developed. Ongoing research points to the integration of senses: eardrum movements and saccades are in some correlation. They are actually ahead of the eye movement, as a form of anticipatory expression [67]. Even more relevant is the finding [68] that motoric expression and perception are in a continuous state of interaction. Moving affords evolutionary advantage. Rhythms of cognitive activity and rhythms of the external world (environment, in a broad sense) are entrained in each motoric expression. As a result, rudimentary epigenetic processes contribute to anticipatory expression (preparation for a possible future [69]). One might not subscribe to the "mechanics" of experiments intended to document the process. Light emitting diode (LED) flashes are different in their particular physics from natural stimuli. But the inference that environmental stimuli and the sampling patterns of the living organism end up in some correlation (Abassi and Gross [70] report on motor-auditory interaction) is justified.

The importance of seeing brings the eye to the forefront. This prompted many questioning minds to look at what it is, how it functions, how to explain the variety through which we experience it—in essence, WHY? questions. The cognitive leap from the eye considered as lens to the eye identified as a neuronal process, and to a statement such as "We see with our brain" is indicative of alternative views informed by the increased empirical evidence of anticipatory-processes characteristics of seeing. Anticipatory seeing, as documented by Berry et al. [71] in studying the anticipation of moving stimuli by the retina, made it clear that processes related

to it are distributed. The research proved that anticipation of moving stimuli begins in the retina.

That genetics describes part of the process is indisputable. It is no longer that we expect the visual cortex to do some heavy extrapolation of trajectory, as in mechanical models that dominated the science of vision (and which continue to flourish since "machines for seeing" are based on it). But we now know that retinal processing, and almost all other vision-related processes are not only in reactions to stimuli, but actually pro-active. Even if pro-activity is not equally distributed along all sensory channels—some are slower in anticipating than others, not the least because sound travels at a slower speed than light does, for example—it defines a characteristic of human perception and sheds new light on motoric activity, itself of anticipatory nature [72].

9 Accounting for Change

Empirical findings concerning vision (for example), or the nature of motoric activity, deserve attention because they document progression from shallow reductionist explanations to deeper and deeper views. The path is from the physicality of the eye (still important to the optometrist, who examines patients for cataracts, glaucoma, macular degeneration, etc.) to its metaphysics. The word is used in its strictest sense: the inquiry into the fundamental nature of reality, the first principles of being, identity and change, space and time, causality, necessity, and possibility. Seeing, or performing an activity, not unlike hearing, smelling, touching, and tasting, are part and parcel of knowing oneself and the world.

To live is to interact with the world. Epigenetics is the knowledge domain that describes the open-ended variety of interactions of genetic consequence. Epigenetic interventions take place within the larger framework of anticipatory processes, which expand beyond epigenetic interventions. They are driven by the survival of life and its creative reproduction.

It is, therefore, necessary to define the nature of what we called interactions.

Axiom 4
To observe the world is to interact with the world.

Corollary 1
To observe is to change the world.

Corollary 2
To observe is to be changed by the world.

Corollary 3
Observations are part of an open-ended cycle of entangled parallel recursions.

How do we account for change? If a witness, i.e., a living entity from another universe that could record change completely disentangled from the world, were possible, it would experience an epistemological conundrum: Being disentangled (sometimes described as objective, unaffected by the observed) ultimately means that the record would be empty. Such a witness, or observer, while conceivable—at least in a description using words, themselves not independent of what they stand for—is rather impossible. In a different context [73], I postulated (paraphrasing [74]) that *One cannot NOT interact.* In a world free of interactions, there is no change to account for, and no need to describe it. All there is is part of the world, and consequently to observe anything in the world is to interact with it. For the sake of simplification, we can separate the changing world (to which the observer belongs) and the observer itself, changing as the world to which it belongs changes. Based upon this simplified model we can consider their interactions (Fig. 9).

Some of these interactions are part and parcel of the dynamics of the world: some random, some regular, some predictable, some unpredictable. Interactions triggered by the actions of observing the world are reflective of the Why? question: Why the succession of day and night? Why warm and cold? Why hard and soft? Why fast and slow? And so on. On top of these particular Why? questions is the WHY? of "Why observe?" Through epigenetic interaction, the lifeless DNA or the genome might become part of the process, but it is not where the answer or answers could be found.

Lifeless matter interactions correspond to the dynamics of change of matter and energy. Living matter interactions are the expression of the self-preservation of life. This is where the immediate answer to the WHY? of "Why observe?" is in plain view: "To maintain life." In other words, in opposition to entropy, resisting decay. The viability domain—between the inception of life and the end—is at the same

Fig. 9 Observing the world and being part of the world

time the domain of the continuous remaking of life. Therefore, epigenetics seems entangled with anticipatory processes driven by the realization of the possible future. At the human level, this is expressed in the postulate "We are what we do." But so is every other living entity, and so are all their constitutive elements, e.g., cells, tissues, organs, etc. You can infer from the whole to each of it, but you cannot infer from the parts to the whole. Reductionism does not cut it! This is even more evident in respect to the DNA—a lifeless crystal, with a unique configuration subject epigenetic action. Inference from the genetic, i.e., chemistry of life, to life processes are always after the fact. All reductionism is by necessity sterile.

10 The Consequential Nature of Foundation Research

The matter-energy interlocking in the living is such that identity is preserved from top to bottom and reinforced from bottom to top. It is not only the individual organism—microbe, yeast, mushroom, worm, spider, cat, elephant, human being—that acts in anticipation—of opportunity, danger, long and short-term changes of all kinds ranging from the day-and-night cycles to catastrophes of all kinds—but each constitutive element. The DNA is fixed: its elaborate double helix structure is meant to preserve it as a whole. The recursive chronicle of successive or simultaneous causal processes experienced via epigenetic interventions, which ultimately change the protein profile of individual organisms, is in itself an expression of observations of self and the medium of existence: *Umwelt*. It is understood as that specific part of the existential reality, i.e., environment, in which everything alive is what it does. Environment integrates the material world—some as living matter, some as non-living matter—and the spiritual. This view is the basis for evaluating the consequential nature of establishing a foundation for a science that integrates the reactive and the anticipatory.

The SAR-Cov-2 virus binds to the receptor human ACE2 (hACE2) through its receptor-binding domain (RBD) and is proteolytically activated by human proteases. In simpler words, a lifeless particle is sucked into the living dynamics of cell activity where copies of the virus are generated. There is reaction to the virus, and there is anticipatory activity. The process documents the anticipatory behavior associated with cell renewal: the self-reproduction guided by the RNA. This example cries for acknowledgment since the entire activity focused on mastering the pandemic focused on an incomplete understanding of epigenetics. Even the spectacular mRNA vaccine, a victory of synthetic biology, reflects this epistemological limitation. Concretely, it is expressed in the worrisome number of breakthrough infections, as well as in the fact that boosters have not diminished the danger of infection (increasing it in some cases). Indeed, to prevent via immune processes, in the sense in which Edward Jenner conceived vaccination, as an anticipatory action, is different from synthetic epigenetic action via the mRNA process. A new booster will not do [75]!

Humans are what they do. The purpose of increasing the number of opportunities transcends the immediateness of preserving life. This often takes place at the

expense of other species, i.e., of nature. Some were totally eliminated; others, such as domesticated animals or hybridized plants, were forced into patterns of existence subordinated to those of the human evolving towards a condition of entitlement. As this behavior becomes part of social life, anticipatory action becomes less beneficial. One example: the perils of all kinds of pests related to domestication with the purpose of multiplying food sources (e.g., avian pest, swine flu, mad cow disease, etc.) is the outcome of increased vulnerability.

Lemma 4

To observe the world is an action in anticipation of its change.

To observe the world is more than to record it; it is to make choices in the present for the possible future.

Movement of lifeless matter (e.g., stars, objects, floating pieces of wood, ions in the brain, nucleotides, etc.) is experienced at a level of observation at which answers to the WHY? (Why is it moving?) depends on the scale of perception. As we have seen, Newton's physics and Einstein's relativity theory are such answers. They are formulated in the language of mathematics and were experimentally tested. The formal encompassing description, which Rosen [22] called the largest model, can be formally processed with the same effectiveness with which what the description (usually a mathematical formula) conveys can be manipulated. This gives physics practical significance: marble can be "mined," cut, and processed; Newtonian physics guides almost the entire operation. For that matter, his physics guides the technology of the Industrial Revolution. Descriptions of energy processes guide the emergence of engines.

The same does not hold true for living matter. Observing cycles of a tree (from the germinating seed to a seedling) has no significance for our understanding of its lower-level change. Observing a fish, a lion, a microbe move in the respective universe of their existence is probably a source of knowledge about that particular movement, but not about cell dynamics, neurons, their physiology or even their anatomy—not to mention the genetic process that extends from inception to death (i.e., over the viability domain).

The self-disrupt behavior of octopuses is rather of the nature of their unique biological identity, but not of their chemical make-up—the genetics—or of their physical properties. Change in living matter is of particular interest (and significance) since it conjures anticipation as an integrated expression that does not imply a "largest" model. There is no such thing as the equivalent of gravity or of relativity in the domain of life. Whether quantum descriptions (non-locality, entanglement, superposition, etc.) are meaningful in describing life is still open to debate. However, probabilistic and stochastic understandings, appropriate for describing the non-living, entail the heavy burden of determinism and therefore miss the non-determinism of anticipatory processes. To be consequential, a theory of life must transcend the arbitrariness of right and wrong and focus on the possible. Without future, there is no life.

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