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Noncanonical Nucleotides in the Genome Around the Maternal-Zygotic Transition

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ABSTRACT

From the very moment of fertilization and throughout development, the cells of animal embryos have to continuously orchestrate the dynamic reorganization of their epigenetic landscapes. One of the earliest major events of this reorganization occurs during the time of the maternal-zygotic transition (MZT), when the control of the developmental process gradually shifts from maternal factors (initially present within the oocytes) to the genes of the embryo itself. As maternal transcripts and proteins are degraded, parental epigenetic information is often erased, and pioneer factors will turn on the transcriptional activity of the zygotic genome. This activation also coincides with the decompaction of the chromatin, which is essential for the successful initiation of gene expression in the zygote. Interestingly, in the past decades numerous studies reported findings that supported the role of noncanonical nucleotides in the process of MZT. These nucleobase moieties in these noncanonical nucleotides are covalently modified versions of the canonical bases, and often show a very dynamic presence within the genome. While most of the recent studies have deciphered in great detail the epigenetic role of methylcytosine and its derivates, other Noncanonical bases have received less attention. Here we suggest that the incorporation of nucleotides from deoxyuridine-triphosphate (dUTP) or 6-methyl-deoxyadenine-triphosphate (6m-dATP) into the genome is not mere noise or replication error but serves a well-defined purpose: to aid chromatin decompaction through the timely induction of DNA repair pathways.

1 | Introduction

One of the most critical periods during the life cycle of a multicellular organism is its early embryonic development. A single fertilized egg cell must develop into a complex, fully functional organism through a series of highly regulated events that coordinate cellular differentiation, tissue formation, and morphogenesis. At the heart of this developmental program lies the maternal-to-zygotic transition (MZT), a crucial period during which control of development shifts from maternally deposited factors to the newly activated zygotic genome (Lee et al. 2014a; Vastenhouw et al. 2019).

The regulation of MZT varies significantly across species, reflecting different evolutionary strategies and developmental constraints. This variation is particularly evident in the timing of zygotic genome activation (ZGA), which occurs through distinct waves of transcriptional activity. While some species, such as zebrafish and *Xenopus*, undergo rapid early cell divisions before major ZGA, others, like mice and humans, activate their zygotic genome much earlier in development (Lee et al. 2014a; Vastenhouw et al. 2019). These differences in developmental timing are intrinsically linked to various molecular mechanisms, including the nuclear-to-cytoplasmic ratio, availability of

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specific transcription factors, and the regulation of cell cycle checkpoints.

During early development, the precise control of DNA replication and chromatin organization is essential for successful embryogenesis. Traditional research has primarily focused on the role of canonical deoxynucleoside triphosphates (dNTPs) in these processes. However, recent technological advances have revealed an additional layer of complexity in the form of Non-canonical DNA bases and their modifications. Accumulating evidence suggests that such modified nucleobases play crucial roles in epigenetic regulation and gene expression control (Febrimarsa et al. 2023; Le Franc et al. 2020; He et al. 2019; McGaughey et al. 2014; Parasyraki et al. 2024; Ross et al. 2020; Ross et al. 2023; Smith and Meissner 2013).

The intersection of developmental timing, genome activation, and the role of DNA modifications in the regulation of gene expression programs presents an exciting new frontier in developmental biology. Understanding how these processes are coordinated and regulated is not only fundamental to our knowledge of embryonic development but could also have important implications for understanding the origins of particular developmental disorders. Here we summarize the current understanding of MZT regulation across different animal species and explore the emerging role of noncanonical nucleotides in development, highlighting recent discoveries and their potential implications.

2 | Regulation of the Maternal-Zygotic Transition in Different Animal Species

As detailed above, a crucial step in the intricate process of early embryonic development is the activation of the zygotic genome: during MZT the control of development gradually shifts from maternally deposited factors to the zygote (Brantley and Di Talia 2024; Lee et al. 2014a; Vastenhouw et al. 2019).

Before the MZT, during the earliest phase of embryonic development, all cellular processes of the embryo are entirely determined by RNAs and proteins inherited from the mother, while the zygotic genome remains inactive. During MZT these factors are gradually degraded, and thereafter the newly transcribed zygotic genes become the main drivers of the developmental process (Tadros and Lipshitz 2009). Incidentally, this process necessitates the erasure of the epigenetic programming of the parental pronuclei, inherited from two highly specialised cell types, the oocyte and the sperm, to (re)establish the totipotency of the zygote (Biechele et al. 2015; Clift and Schuh 2013; Du et al. 2022; Lee et al. 2014b; Messerschmidt et al. 2014; Zhang and Xie 2022).

Despite being fundamental to both plant and animal embryogenesis (Baroux et al. 2008), the exact molecular mechanisms governing MZT are still not fully understood and subject to extended research programs. For example, it is still debated how exactly the timing of embryonic genome activation is regulated.

In most species, zygotic genome activation (ZGA) occurs in two waves: usually the earliest signs of transcription (the so-called "minor wave") will precede the bulk of transcriptional activity ("major wave"). The timing of MZT relative to the number of cell divisions the embryo completed differs widely between animals with different breeding strategies (Figure 1). Broadly speaking, in anamniote vertebrates (e.g. fish and amphibians), where fertilization is external and embryos are exposed to their environment from the earliest stage, right after fertilization we can witness a series of rapid, synchronous cell cycles, whereas amniote embryos, protected during early stages of development, perform slow divisions from the very beginning of their development. The fury of cell divisions that characterizes anamniote development will come to an end only at the midblastula transition (MBT), which coincides with the major wave of ZGA in the species examined. This transition occurs at the 10th and the 12th cell cycle in the zebrafish (*Danio rerio*) and the African clawed frog (*Xenopus laevis*), respectively, whereas the major wave of ZGA occurs as early as the second cell cycle in chicken (*Gallus domesticus*) and mouse (*Mus musculus*), and at 4–8 cells stage in human (*Homo sapiens*) embryos (Jukam et al. 2017; Rengaraj et al. 2020). Insects follow a developmental pattern similar to that of anamniotes, and MBT and major wave of ZGA co-occur in fruit flies (*Drosophila melanogaster*) at nuclear cycle 14 (Harrison and Eisen 2015; Kwasnieski et al. 2019). In contrast, in sea urchins (*Strongylocentrotus purpuratus*) and the nematode *Caenorhabditis elegans*, ZGA occurs shortly after fertilization (Andéol 1994; Robertson and Lin 2015). *Hydractinia* embryos show a ZGA pattern more similar to fast-developing Bilaterians, where the activation of the nuclear genome starts around the 64-cell stage, during the early stages of gastrulation (Ayers et al. 2023).

In fish, amphibians and *Drosophila* where fast cleavage precedes ZGA, a commonly accepted regulator that contributes to the activation of the zygotic genome is the nuclear-to-cytoplasmic ratio ("N/C ratio"). Cleavage cycles in these species are missing the G1 and G2 phases, thus there is no cytoplasmic growth before MBT. Consequently, each cell division essentially doubles the N/C ratio, and results in an exponential decrease of the concentration of particular factors (e.g. histones, transcription factors, or replication factors) compared with the amount of genomic DNA. In this model, when a particular threshold is reached, MBT and major wave of ZGA will occur (Brantley and Di Talia 2024; Chen et al. 2019; Collart et al. 2013; Jevtić and Levy 2017; Joseph et al. 2017; Kane and Kimmel 1993; Shindo and Amodeo 2021). It needs to be pointed out, however, that gradual accumulation of particular factors can also reach threshold values over time, and indeed, in zebrafish, the accumulation of Nanog, SoxB1/Sox19b and Pou5f3 transcription factors, or that of Brd4 and P300 chromatin modifiers seems to be sufficient to drive ZGA (Chan et al. 2019; Lee et al. 2013; Pálfy et al. 2020; Veil et al. 2019).

Besides the N/C ratio, depletion of the maternal deoxynucleoside triphosphate (dNTP) pool also appears to have a role in MZT, at least in some species (Djabrayan et al. 2019; Liu and Großhans 2019; Liu et al. 2019; Song et al. 2017). In *Drosophila* embryos only about half of the dNTP pool necessary to complete cleavage is available at fertilization. The rest of the dNTPs will be synthesized through ribonucleotide reductase (RNR) activity (Figure 2A) (Liu et al. 2019; Song et al. 2017). If dNTP levels are kept artificially high, both deceleration of fast cell

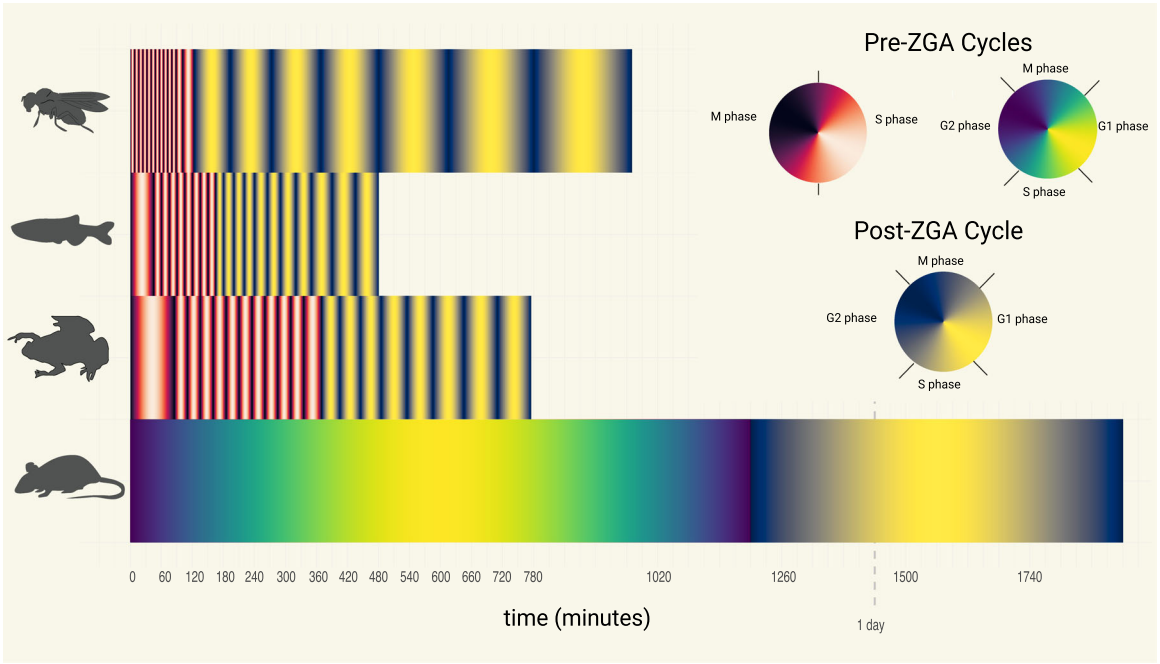

FIGURE 1 | Timing of ZGA in different model species. In *Drosophila* and anamniote species, like zebrafish and *Xenopus* a series of fast cell divisions are observed prior ZGA, whereas in mouse embryos ZGA occurs immediately after the first cell-cycle, which is extra-long. Fast dividing animal embryos undergo a special form of cell division (often termed cleavage) which lacks G1 and G2 phases. In contrast, the first cell division of mouse embryos shows all the typical cell phases. (Based on: Edgar and O'Farrell 1989; Flach et al. 1982; Kane and Kimmel 1993; Newport and Kirschner 1982).

cycles and ZGA will be impaired in *Drosophila* embryos, ultimately leading to developmental failure (Djabrayan et al. 2019).

The role of cell cycle checkpoints in the control of DNA replication has been also implicated in the regulation of ZGA in multiple species. Activation of the serine/threonine kinase Checkpoint kinase 1 (Chk1) by phosphorylation is a key step in the DNA damage response in Metazoans, and a burst in Chk1 activity often coincides with MBT. In the *Drosophila* maternal-effect *grapes* mutants, which lack the fly homologue of Chk1, early syncytial divisions occur normally, but MBT fails (Fogarty et al. 1997; Sibon et al. 1997). Zebrafish show differential activation of DNA-damage pathways pre- and post-MBT, and Chk1 becomes active only around MBT and its precocious activation lengthens the cleavage-specific fast replication cycles (Zhang et al. 2014). In *Xenopus* embryos, Chk1 is also activated transiently at MBT (Shimuta 2002), but ectopic activation of the kinase can lead to slower cell cycles even pre-MBT (Conn et al. 2004). Intriguingly, this effect can be also replicated by the injection of linear dsDNA, mimicking DNA damage (Brantley and Di Talia 2024; Conn et al. 2004).

Finally, the absence of factors that facilitate chromatin reorganization during MZT, such as the cohesion subunit Rad21 in zebrafish, or Zelda in the case of *Drosophila*, can also delay the onset of ZGA significantly (Hug et al. 2017; Liang et al. 2008; Meier et al. 2018).

In summary, available evidence suggests that multiple mechanisms contribute to the timely occurrence of MBT and ZGA in diverse species, and while activation by threshold N/C ratios is the dominant process in most examined models, other mechanisms, such as the activation of the Chk1-dependent cell cycle checkpoint and the reorganization of chromatin also contribute to this process.

3 | Noncanonical dNTPs in Development

Over the past decades, research into the regulation of cell proliferation and related DNA replication during the earliest stages of development mostly focused on the incorporation of canonical dNTPs (i.e. dATP, dCTP, dGTP, and dTTP) into the genome. While the presence of noncanonical bases (Figure 3), such as 5-methylcytosine (5mC), in animal DNA was established during the 1950s and 1970s (Vanyushin et al. 1970; Wyatt 1950; Wyatt 1951), it took considerable time to develop the analytical tools and methods that later allowed their reliable detection, necessary to understand their role in gene regulation (Galashevskaya et al. 2013; Lister et al. 2009; Surányi et al. 2023; Tse et al. 2021). Concentrated efforts during the past twenty years uncovered the epigenetic role of methylcytosine and its derivatives, which resulted in a deeper understanding of the enzymatic processes that regulate their presence. Less attention was paid, however, to the presence of base modifications and possible incorporation of other noncanonical dNTPs during the early stages of development, and the enzymatic modification of canonical nucleotides into less canonical forms. As sophisticated new methodologies were developed to accurately measure the amount and position of these relatively rare bases, interest has also grown in understanding the role they can play in the regulation of developmental events (Table 1) (Békési et al. 2021; Galashevskaya et al. 2013; Horváth and Vértessy 2010; Lister et al. 2009; Meng et al. 2024; Pálinkás et al. 2020; Róna et al. 2016; Surányi et al. 2023; Tse et al. 2021).

Noncanonical bases of the DNA, such as 5mC, 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), uracil (U), 5-hydroxymethyluracil (5hmU), 8-oxo-7,8-dihydroguanine (8-oxoG) or N6-methyladenine (6mA) expand our understanding of gene regulation complexity and genetic information interpretation within cells. The tight regulation of gene expression through the activation and silencing of specific genes in different cell types is essential for the development and functioning of multicellular organisms. This regulation is facilitated by the presence of these

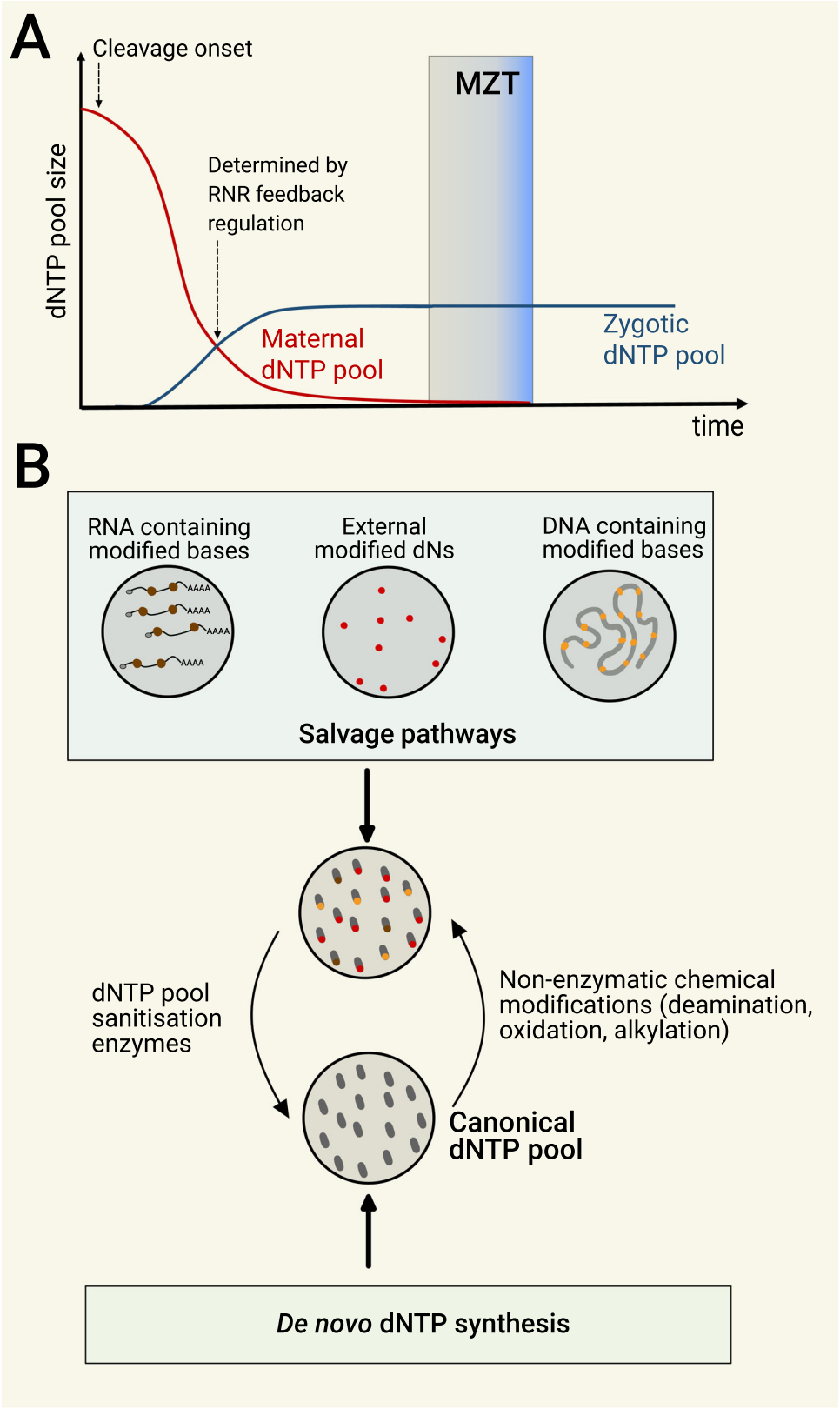

FIGURE 2 | Legend on next page.

noncanonical bases, which contribute to the diverse interpretations of the genetic code, see for example the emerging role of 5hmC in the function of specialised cells, such as neurons (Chen et al. 2017; Hahn et al. 2013; Kriaucionis and Heintz 2009; Mellén et al. 2012; Song et al. 2011; Szulwach et al. 2011).

We posit that identifying noncanonical bases that are often incorporated into the DNA and uncovering their role will provide valuable insights into the mechanisms governing gene expression and cell differentiation, with the potential of uncovering hitherto unsuspected mechanisms of genomic regulation (Carell et al. 2018; Zhu et al. 2018).

3.1 | Role of Cytosine Methylation in Regulating Gene Expression and Methylation-Demethylation Cycles During Early Development

Over the past decades, the methylation-demethylation cycle of cytosines in DNA has emerged as one of the most fundamental mechanisms of epigenetic regulation during vertebrate development and differentiation (Figure 4). During the methylation phase of such cycles, a methyl group is added to the cytosine base of DNA, predominantly at CpG dinucleotides (mCG), resulting in 5mC. This process is chiefly mediated by DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosylmethionine to the 5th carbon atom of the cytosine ring (Lyko 2018). The concentrated presence of 5mC residues is a marker of heterochromatin, and in gene promoters generally leads to transcriptional repression by hindering the binding of transcription factors and attracting proteins that compact chromatin, thus restricting access to the transcriptional machinery (Jones 2012; Mattei et al. 2022). During early development, the establishment of adequate methylation patterns is critical for cellular differentiation, and through the silencing of repetitive elements, cytosine methylation is also essential for genome stability. The reversible nature of this modification also allows for dynamic changes in gene expression, making it an essential mechanism for fine-tuning the genetic program across various developmental stages (Bogdanovi and Gomez-Skarmeta 2014; Greenberg and Bourc'his 2019; Smith and Meissner 2013). These characteristics make cytosine methylation a powerful and complex mechanism in the regulation of gene expression throughout development and/or during the transcriptional response to environmental factors.

During cell divisions, the replication of a methylated DNA sequence creates hemimethylated CpG dinucleotides, where methylation is present only on the original strand. Hemimethylated CpGs attract the maintenance methyltransferase DNMT1, which methylates the newly synthesized unmethylated strand to restore complementary DNA methylation. In contrast, the *de novo* methyltransferases DNMT3A and DNMT3B methylate CpGs that are unmethylated on both DNA strands, converting cytosine to 5mC (Cantone and Fisher 2013; Greenberg and Bourc'his 2019; Seisenberger et al. 2013; Stewart et al. 2016).

The mechanism of the active removal of 5mC methylation marks has remained enigmatic until the discovery of Ten-eleven translocation (TET) enzymes. These enzymes can oxidize 5mC first into 5hmC, then further convert it to 5fC, and finally 5caC. Both 5fC and 5caC can be excised by the thymine DNA glycosylase (TDG) enzyme and replaced with unmethylated cytosine by the base-excision repair (BER) molecular machinery (Cantone and Fisher 2013; Greenberg and Bourc'his 2019; Seisenberger et al. 2013; Stewart et al. 2016).

The epigenetic role of cytosine methylation in animal embryogenesis, which was first extensively studied and deciphered in mice (Li et al. 1992; Monk et al. 1987), appears to be conserved across vertebrate species (Al Adhami et al. 2022; Jiang et al. 2014). For example, in zebrafish embryos cytosine methylation is also vital for epigenetic regulation and chromatin organization, significantly influencing early embryonic development (Akdogan-Ozdilek et al. 2020; Chernyavskaya et al. 2017).

In mammals, a characteristic reset of global methylation patterns occurs right after fertilization. This (almost) global erasure of paternal methylation allows the embryo to reset and establish a new epigenetic landscape that is compatible with totipotency and early development, while still retaining key regulatory regions, such as imprinted genes (Greenberg and Bourc'his 2019; Smith and Meissner 2013).

Given the generally conserved role of cytosine methylation in the regulation of gene expression, it came by surprise to see that

FIGURE 2 | DNA precursor availability during early embryogenesis. (A) During early embryogenesis, the initial supply of deoxynucleoside triphosphates (dNTPs) is provided by maternally deposited stores. However, this maternal contribution is insufficient to meet the full demand until the major zygotic genome activation (ZGA) (Liu and Großhans 2019; Liu et al. 2019; Mathews 1975; Song et al. 2017). Enzymes involved in dNTP synthesis are among the first to be activated during embryogenesis, well before ZGA. Additionally, maternally deposited transcripts play a crucial role in supporting early dNTP production (Pérez-Mojica et al. 2024). After a species-dependent number of cell divisions, the concentration of maternally deposited dNTPs decreases to a level that allows for the activation of ribonucleotide reductase (RNR). RNR is the key enzyme for *de novo* dNTP biosynthesis, which regulates the dNTP pool in a species-specific manner through allosteric control. (B) In addition to the four canonical DNA precursors – dATP, dGTP, dTTP, and dCTP – the cellular dNTP pool contains varying amounts of noncanonical dNTPs. These molecules may arise through different salvage pathways, including the recycling of modified RNA bases that become incorporated into DNA (Febrimarsa et al. 2023; Li et al. 2021). It has been shown that under experimental conditions external nucleosides could be taken up by embryos and then incorporated during early divisions (Mathews 1975). The *de novo* dNTP pool is also vulnerable to chemical modifications from reactive agents in the cellular environment. Notably, the cellular dNTP pool is several orders of magnitude more susceptible to modification than the DNA molecule itself (Rudd et al. 2016). Finally, the composition of noncanonical dNTPs is influenced by both the availability and efficiency of enzymes responsible for sanitizing the dNTP pool.

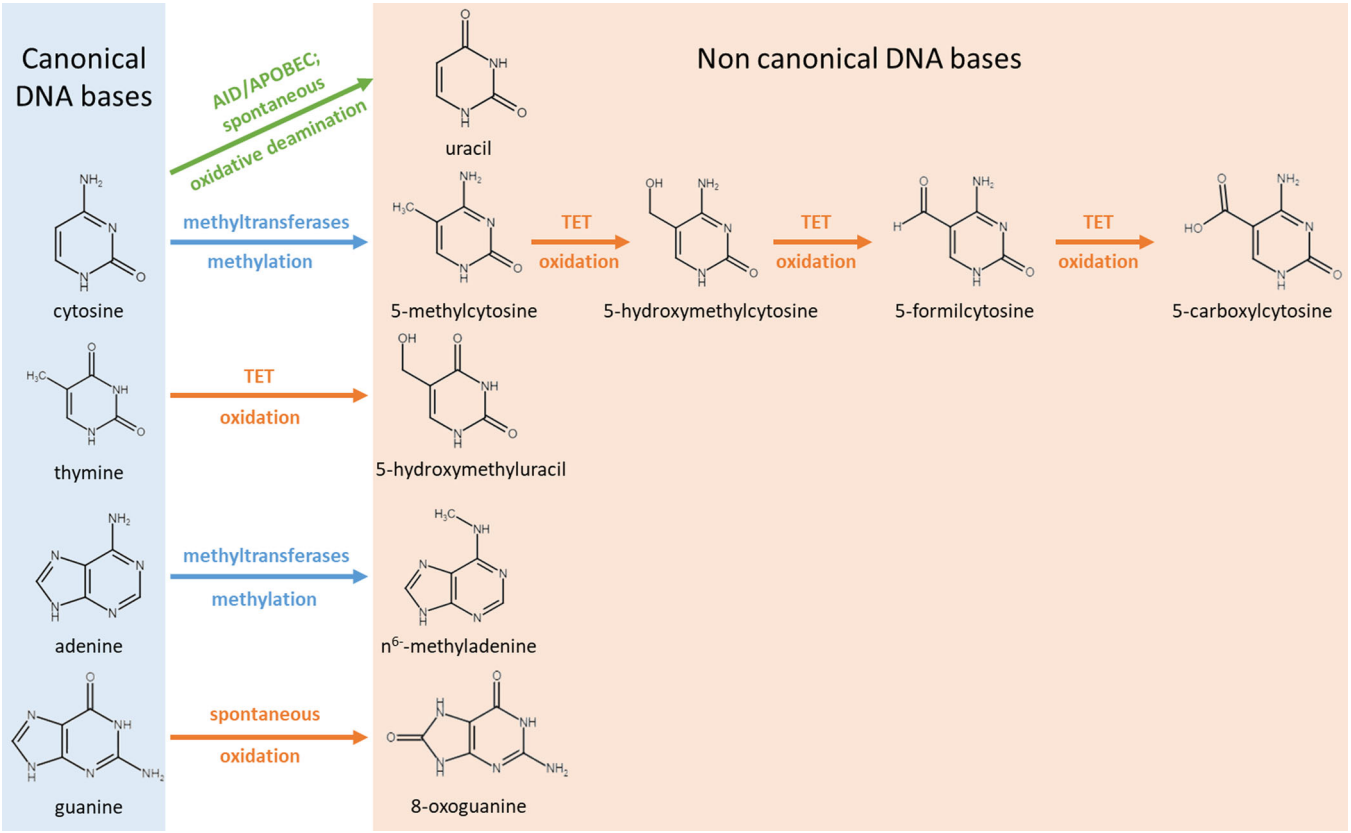

FIGURE 3 | Chemical structure of canonical and noncanonical nucleotides detected in genomic DNA. The relevant chemical processes, and, if relevant, the names of the contributing modifying enzymes are indicated above the arrows.

the methylation dynamics of the genome at the time of ZGA in teleosts significantly differs from that seen in mammals (Bogdanovi and Gomez-Skarmeta 2014; Hackett and Surani 2013). Research on DNA methylation patterns in zebrafish has demonstrated that sperm methylation profiles closely resemble those observed at ZGA, highlighting the paternal genome's role in establishing chromatin states crucial for early embryonic development (Jiang et al. 2013; Potok et al. 2013). Conversely, the zebrafish oocyte exhibits extensive methylation of genes important for germline functions and early development, and these genes undergo demethylation during the zygotic and cleavage stages. A similar dynamic was recently found in medaka as well (Ross et al. 2023).

Inhibition of DNMTs in zebrafish has further underscored the significance of cytosine methylation in silencing specific genes and chromatin groups during ZGA. Impaired cytosine methylation during early development results in premature and erroneous gene expression at early stages, resulting in altered embryogenesis (Martin et al. 1999; Potok et al. 2013; Shirai et al. 2023). Zygotic inactivation of *dnmt1* can also lead to activation of retrotransposons (Chernyavskaya et al. 2017). An interesting pattern of non-CpG methylation (mCH) can be also observed at satellite repeats both in zebrafish and medaka embryos. The dynamics of mCH in these species mirror that of the mCG, with a decrease observed around MZT (Ross et al. 2020; Ross et al. 2023).

Lately, a potential role for 5fC, the oxidized form of 5mC has also emerged both in amniotes and anamniotes. Both in *Xenopus* and mammalian embryos an enrichment of genomic 5fC sites can be observed around ZGA, and new evidence suggests that this might be linked to the activation of Pol III target tRNA genes (Gao et al. 2020; Parasyraki et al. 2024; Zhu et al. 2017).

The presence and distribution of cytosine methylation, along with the methylation toolkit, exhibit significant variability throughout the animal kingdom (Lyko 2018; de Mendoza et al. 2020; Vogt 2022). Initial investigations into cytosine methylation in invertebrates predominantly focused on widely used laboratory model organisms like *Drosophila melanogaster* and *Caenorhabditis elegans*, which originally raised the possibility that, unlike vertebrates, invertebrates have lowly methylated genomes with sporadic and mosaic methylation (Vogt 2022). 5mC methylation is not observed in the flour beetle (*Tribolium castaneum*) and *Drosophila melanogaster* (Zemach et al. 2010) genomes, despite the fact that in beetles *Dnmt1* is an essential gene (Schulz et al. 2018). *Caenorhabditis elegans* has long been believed to possess DNA free of 5mC, but more sensitive methods led to the discovery of trace amounts of cytosine methylation in this organism (Hu et al. 2014).

More recent research, however, highlighted that observations in these model organisms cannot be generalized to other invertebrates. In fact, invertebrate species are highly diverse regarding the methylation levels and methylation patterns of their genome. Some organisms in fact show methylation features that are reminiscent of those found in vertebrates: for example, in the sponge *Amphimedon queenslandica* more than 80% of CpGs

TABLE 1 | A summary of the known roles for noncanonical nucleotides in development and beyond.

Nucleobase	Role at ZGA	Other/general role during embryonic development	Other roles
5mC and modifications	Silencing specific genes and chromatin groups (Martin et al. 1999; Potok et al. 2013; Shirai et al. 2023) Enhancing the Pol III-dependent transcription of tRNA genes (Parasyraki et al. 2024).	Critical for cellular differentiation and essential for genome stability (Bogdanovi and Gomez-Skarmeta 2014; Greenberg and Bourc'his 2019; Smith and Meissner 2013). Epigenetic regulation and chromatin organization (Akdogan-Ozdilek et al. 2020; Chernyavskaya et al. 2017)	A marker of heterochromatin; when present in gene promoters generally induces transcriptional repression (Jones 2012; Mattei et al. 2022)
6mA	In *H. symbio-longicarpus* early embryos: in genome triggers the activation of Alkbh1, which seems to be essential for the timely initiation of ZGA in this animal (Febrimarsa et al. 2023)	Not reported	The genomic presence of 6mA is still debated (Boulias and Greer 2022). Potential role in transposon activation and aging (Sturm et al. 2023a; Sturm et al. 2023b).
U	Not reported	Genomic uracil levels show a dynamic profile during the later stages of *Drosophila* larval development (Muha et al. 2012; Starczak et al. 2022)	Neutralization of foreign ssDNA: Cytosine deamination serves as a defense mechanism by marking foreign single-stranded DNA for degradation or mutational inactivation (Stenglein et al. 2010) Generation of diversity in adaptive immune responses: In processes like somatic hypermutation and class switch recombination, error-prone repair mechanisms act on deaminated cytosines (Çakan and Gunaydin 2022; Chi et al. 2020)
8-oxoG	Not reported	Not reported	In promoters: stimulate transcription factor binding (Poetsch et al. 2018) Telomer organization (Rosa et al. 2021) Formation of structural elements (e.g. G-quadruplexes) (Roychoudhury et al. 2020)

are methylated, with a strong reduction at promoters (de Mendoza et al. 2019).

Interestingly, in contrast to vertebrates, most invertebrates apparently do not (or just moderately) methylate transposable elements, as was demonstrated in the marbled crayfish (*Procambarus virginalis*) (Gatzmann et al. 2018), the European honeybee (*Apis mellifera*) (Feng et al. 2010; Zemach et al. 2010) the tunicate *Ciona intestinalis*, the silk moth (*Bombyx mori*), and the sea anemone (*Nematostella vectensis*) (Zemach et al. 2010). Counterexamples, however, have also been found, like certain nematodes (Rošić et al. 2018). Gene body methylation on the other hand is more conserved across Bilaterian taxa, and it is associated with elevated transcription (de Mendoza et al. 2020; Sadler 2023) and prevents aberrant transcription initiation (Männer et al. 2021; Neri et al. 2017). In invertebrates, these modifications remain constant during development and among cell types, therefore they do not participate in environmental adaptation (Feng et al. 2010; de Mendoza et al. 2020; Xu et al. 2019). In contrast, mammalian cytosine methylation, as discussed earlier, is much more dynamic and undergoes two major waves of global 5mC reprogramming (Lee et al. 2014b).

Of note, in eusocial insects, 5mC was postulated to play a role in caste determination (Bonasio et al. 2012; Lyko et al. 2010), but this hypothesis remains under investigation (de Mendoza et al. 2020). The ants *Camponotus floridanus* and *Harpegnathos saltator*, just like the honeybee have lowly methylated genomes, with 5mC marks primarily found in exons (Bonasio et al. 2012).

While cytosine methylation has emerged as the paradigmatic epigenetic modification regulating gene expression during early

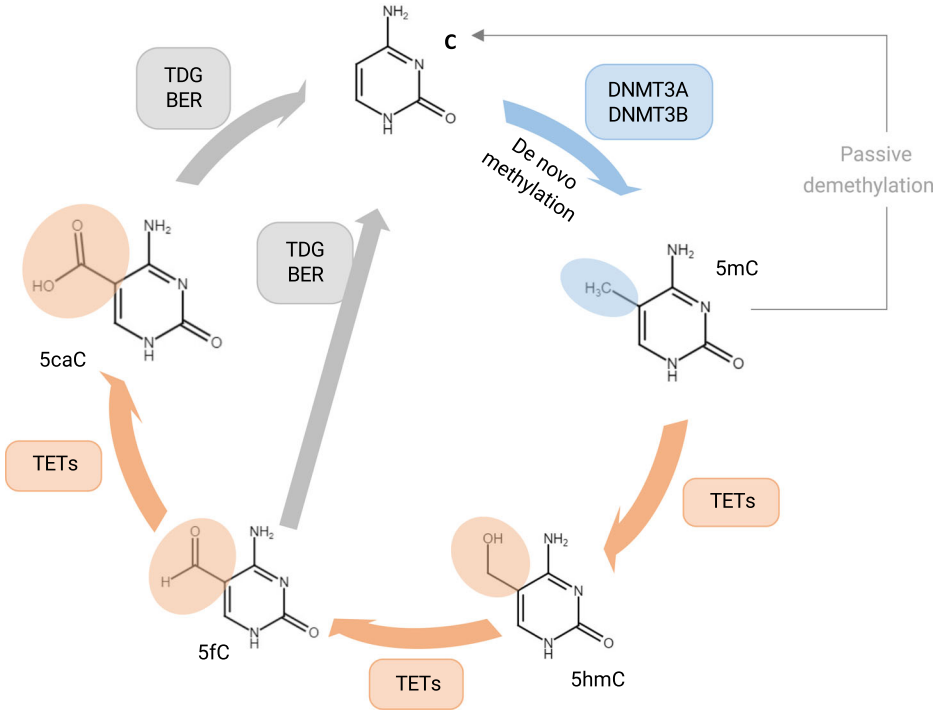

FIGURE 4 | Methylation and demethylation pathways regulating cytosine methylation. DNA methyltransferases (DNMTs) catalyse the methylation of cytosine by positioning the methyl group at the C5 position. Ten to eleven translocation (TET) enzymes can catalyse the oxidation of 5mC to 5hmC. Further TET-dependent sequential oxidative reactions lead to the conversion of 5hmC to 5fC and 5caC. Recognition and removal of 5fC and 5caC is carried out by thymine-DNA glycosylase (TDG) and the generated abasic site is repaired via the base excision repair (BER) mechanism, thereby generating an unmodified cytosine. The "dilution" of modified cytosines (5mC or 5hmC) during DNA replication can also result in unmodified cytosine by a mechanism called passive DNA demethylation, which can be TET-independent or TET-assisted.

development, recent research has also highlighted the potential epigenetic significance of other noncanonical nucleobases, such as uracil, 5hmU, 8-oxoG and 6mA.

3.2 | Detection of 6mA in Early Development

Despite being a common type of DNA modification in prokaryotes and protists, the presence and epigenetic relevance of 6mA in animals is highly debated (Boulet et al. 2023; Boulias and Greer 2022; Feng et al. 2024; Greer et al. 2015; Kong et al. 2022; Li et al. 2022a; Schiffers et al. 2017; Sturm et al. 2023a; Wu et al. 2016; Zhang et al. 2015; Zhang et al. 2023). Analytically sensitive methods failed to find significant amounts of 6mA in nuclear DNA, which led many to question if this modification is a stable component of the epigenetic landscape in metazoans. Several research groups came to the conclusion that genomic 6mA is present due to the erroneous incorporation of N6-methyladenosine (m^6A) derived from the degradation of mRNA (Carell et al. 2018; Febrimarsa et al. 2023).

The role of m^6A in regulating mRNA stability and translation has been deciphered in great detail over the past years. Several high-quality reviews have been published to describe the function of m^6A, the most abundant mRNA modification in eukaryotes, and we refer the readers to them for a comprehensive overview of m^6A function under different contexts (Boo and Kim 2020; Deng et al. 2023; Hamar and Varga 2023; Livneh et al. 2020; Meyer and Jaffrey 2014; Meyer and Jaffrey 2017; Sendinc and Shi 2023; Widagdo et al. 2022; Zaccara et al. 2019). Here we just want to highlight those papers that survey m^6A dynamics during the early stages of development. In vertebrates, a significant proportion of maternal transcripts carry m^6A, which accelerates their programmed degradation during MZT, through an evolutionarily conserved mechanism (Du et al. 2015; Ivanova et al. 2017; Kontur et al. 2020; Zhao et al. 2017). Interestingly, while the m^6A-dependent degradation of maternal mRNAs is also observed in *Drosophila* embryos, in this case, the molecular players involved in the process appear to be different (Zhang et al. 2022). Elevated levels of transcripts containing m^6A during early development have also been reported in animals as diverse as mice (Wu et al. 2022), the fall armyworm (*Spodoptera frugiperda*) (Chen et al. 2023), the oyster *Crassostrea gigas* (Le Franc et al. 2020) more recently in the cnidarian *Hydractinia symbiolongicarpus* (Febrimarsa et al. 2023).

Levels of m^6A in RNA decrease dramatically in all observed species around ZGA, mostly due to the clearance of maternal products (Aanes et al. 2019; Febrimarsa et al. 2023; Le Franc et al. 2020; Lence et al. 2016; Zhao et al. 2017; Zhu et al. 2023). This observation also suggests that cytoplasmic levels of m^6ATPs increase in parallel with the degradation of maternal transcripts and through the nucleotide salvage pathways. This pool might be a source for the misincorporation of 6mA during DNA replication as well, during the cleavages that precede ZGA (Figure 2B). This model has been put forward recently to explain the dynamics of 6mA in the genome of *Hydractinia* embryos (Febrimarsa et al. 2023). In these developing cnidarian

embryos, genomic 6mA is slightly above background level in the genome during the very first stages, then rapidly accumulates and peaks at the 16-cell stage, a dynamic that is complementary to that observed for m^6A in RNAs. The distribution of 6mA appears to be random, implying no epigenetic function, and by the 64-cell stage, which is the time of the ZGA in this organism, the amount of genomic 6mA drops back to background levels. Of note, the knock-down of alkB homolog 1, histone H2A dioxygenase (Alkbh1), an important initiator of m^6A-clearance, results in higher genomic m6A levels compared to wild-type at the 64-cell stage, and causes delayed ZGA (Febrimarsa et al. 2023).

Cytoplasmic m^6A as the source of (misincorporated) 6mA has also been suggested in the case of human and mouse cell lines where the low but consistent levels of genomic 6mA seem to originate from the conversion of m^6A derived from RNA degradation (Musheev et al. 2020).

Dynamic changes in genomic 6mA levels have also been observed during the early embryogenesis of zebrafish, pig (Liu et al. 2016), and fruit fly (He et al. 2019; Zhang et al. 2015) embryos, too, with a significant temporary enrichment right before ZGA. Another study reported a seemingly conflicting result, showing a linear increase in genomic 6mA content in mouse and zebrafish genomes until reaching adult levels (Fernandes et al. 2021). However, because this study's temporal resolution differed from that of previous studies focused on early development, a direct comparison is not appropriate. Notably, the entire timeframe examined in previous studies fell within the range of the zero and first time points of this study. Comparison of various studies is also burdened by various methodological issues thoroughly addressed in (Lyu et al. 2023). Unlike in *Hydractinia* embryos, where 6mA incorporation in the genome appears to be random, available data suggests nonrandom incorporation in bilaterian embryos. In both *Drosophila* and zebrafish 6mA is enriched in repeat elements. In zebrafish embryos, a small enrichment around transcriptional start sites and exons has been also detected, while in the case of fruit fly embryos a similarly small but significant enrichment was observed in some coding genes (He et al. 2019; Liu et al. 2016). One of the genes enriched for 6mA in the early *Drosophila* embryo is *zelda*, which encodes a key pioneer transcription factor of *Drosophila* ZGA. Jumu, a Fox-family protein, preferably binds DNA with 6mA and negatively regulates the expression of genes like *zelda*, which suggests a more complex role of 6mA in the ZGA of *Drosophila* (He et al. 2019).

3.3 | Uracil Nucletotides (UMP, dUMP) in the Genome

UMP is a well-known nucleotide in RNA, and despite conventional wisdom, dUMP can also regularly be detected in DNA in small quantities. The widely accepted "RNA world" hypothesis implies that uracil was the "original" base that evolved to pair with adenine (Higgs and Lehman 2015) rendering thymine's evolutionary appearance seemingly dispensable, particularly if the extra methylation step's additional energy cost is taken into account. Yet, with the exception of certain bacteriophages (Kiljunen et al. 2005; Nagy et al. 2021; Takahashi and Marmur 1963), natural selection preferred thymine over uracil as adenine's complementary base in genomic DNA.

The generally accepted explanation for the replacement of uracil with thymine in the DNA is the spontaneous process of cytosine deamination. The hydrolytic deamination of cytosine leads to uracil formation, and this conversion is mutagenic due to the altered base-pairing properties. If left unrepaired, this change leads to C:G to T:A transitions during replication in the newly synthesised strands, therefore, correcting this mismatch is critical. The enzymatic challenge lies in the discrimination between uracils paired correctly with adenine and those erroneously formed from cytosine deamination. The evolutionary solution appears to be the methylation of uracil to form thymine, thus ensuring the accurate discrimination of these bases. RNA, with its shorter lifespan, faces an insignificant threat from cytosine deamination, and is able to fulfill its transient role without the need of instant repair (Vértessy and Tóth 2009).

Initial research on the basal levels of uracil in the DNA of normal human cells provided mixed results (Galashevskaya et al. 2013; Mashiyama et al. 2008; Olinski et al. 2010). More sophisticated analytical techniques have been recently developed (Békési et al. 2021; Horváth and Vértessy 2010; Pálinkás et al. 2020; Róna et al. 2016). These provided an estimate for genomic uracil levels around 400-600 uracils per diploid genome in repair-proficient cells (Krokan et al. 2014).

In living organisms, there are two known ways for uracil to appear in the genome: either by the aforementioned cytosine deamination (spontaneous or enzymatic) or by promiscuous polymerase activity. Most DNA polymerases cannot differentiate between uracil and thymine, so the relative availability of their corresponding nucleoside triphosphates dUTP and deoxythymidine triphosphate (dTTP) determines which is more likely to participate in the reaction (Vértessy and Tóth 2009). Even though dUTP misincorporation is not inherently mutagenic owing to the functional equivalency between uracil and thymine, elevated dUTP misincorporation caused by a perturbed dUTP:dTTP ratio can pose a serious threat to the cell by overactivating the repair machinery, which introduces abasic sites (AP sites) and single-stranded breaks (SSBs) into the genome (Chon et al. 2019; Vértessy and Tóth 2009). This phenomenon has been exploited in various anticancer and antimicrobial therapies by targeting thymidylate biosynthesis or other enzymes responsible for sustaining the appropriate dUTP:dTTP ratio (Nyíri and Vértessy 2017; Roberts and LaBonte 2023).

While cytosine deamination directly jeopardizes genomic integrity (Visnes et al. 2009), interestingly, certain organisms developed specific enzymes to catalyze this mutagenic reaction, under specific conditions. For example, this mutagenic process is exploited in both the innate and the adaptive immune responses of vertebrates (Chen and Wang 2014; Harris et al. 2003). In the former case, cytosine deamination is used to neutralize foreign ssDNA (Stenglein et al. 2010), while in two mechanisms involved in adaptive immune responses, namely somatic hypermutation and class-switch recombination, error-prone repair mechanisms follow the deamination to generate diversity (Çakan and Gunaydin 2022; Chi et al. 2020).

Previous studies have demonstrated that genomic uracil levels show a dynamic profile during the later stages of *Drosophila* larval development (Muha et al. 2012; Starczak et al. 2022), but we have less information about earlier, pre-MZT stages. However, canonical dNTP levels decrease dramatically during cleavage stages, which makes the misincorporation of non-canonical dNTPs more likely during these fast divisions (Figure 2) (Liu et al. 2019).

3.4 | The Origins and Accumulation of 8-oxoG in the Genome

8-oxoG, a common oxidative modification of guanine, is one of the most abundant oxidized bases, occurring at a frequency of 1000 to 10 000 lesions per cell per day in the human genome (Lindahl 1993; Nakabeppu 2014; Ohno et al. 2006; Tubbs and Nussenzweig 2017). 8-oxoG can also be incorporated from the corresponding oxo-dGTP when this is not efficiently sanitized by the MutT Homolog 1 Nucleoside Triphosphate Pyrophosphatase (MTH1) (Mishima et al. 2004). During DNA replication, 8-oxoG can mispair with adenine via a Hoogsteen base pair introducing a mutagenic potential to the cell (Wood et al. 1992). To mitigate this mutational burden, two specific DNA glycosylases have evolved within the base excision repair pathway to repair different consequences of oxoG appearance (Li et al. 2022b; Michaels and Miller 1992). 8-oxoG itself is recognized by 8-Oxoguanine DNA Glycosylase (OGG1), while MutY DNA Glycosylase (MUTYH) removes adenine base opposite from 8-oxoG.

Although, this is a spontaneously occurring modification, and no specific enzymes are known that can catalyze this reaction, the genomic 8-oxoG patterns were found reproducibly detectable suggesting potential regulatory functions (Ohno et al. 2006). Such patterns might be generated by a differential repair efficiency that is influenced by the chromatin compaction and also protective effects of some DNA binding factors (Giorgio et al. 2020; Moore et al. 2016). Recently, several sequencing methods have been developed that are capable to describe the genome-wide distribution of 8-oxoG [AP-seq (Poetsch et al. 2018), OG-seq (Ding et al. 2017), OxiDIP-seq (Amente et al. 2019)], some of them with nucleotide resolution, like Click-Code-Seq (Wu et al. 2018).

Detected 8-oxoG patterns showed correlation with repetitive regions and transposable elements, and also with open chromatin structures marked by H3K9ac and H3K4me2, while 8-oxoG was rather depleted in functional parts of genes such as promoters, termination sites, and coding sequences (Poetsch et al. 2018). In other studies, however, 8-oxoG was found enriched in promoters and untranslated regions (UTRs) preferentially in GpG context (Ding et al. 2017). 8-oxoG accumulation also showed overlap with γH2AX ChIP-seq signals at DNA replication origins found within gene bodies of transcribed long genes or in fragile promoters (Amente et al. 2019; Gorini et al. 2020). Moreover, further studies revealed functional role of oxoG in telomer organization (Rosa et al. 2021), and the formation of structural elements, such as G-quadruplexes (Roychoudhury et al. 2020). It is also well documented that oxoG bases located in promoter elements can interfere with and even stimulate transcription factor binding (Amente et al. 2010; Moore et al. 2016; Müller and Khobta 2021; Pan et al. 2016; Park et al. 2019; Ramon et al. 1999) or nuclear receptor functions (Park et al. 2019; Perillo et al. 2008). Such regulatory roles of 8-oxoG might be achieved through its consecutive repair (Hao et al. 2018), but in some cases the effects are independent from the 8-oxoG repair system (Obermann et al. 2024).

The accumulation or direct regulatory roles of 8-oxoG in genomic DNA during early embryonic stages were not yet investigated despite the available potent experimental tools. Nevertheless, as the high metabolic activity, rapid cell divisions, and the unmatured DNA-repair system make the developing embryos likely more sensitive to oxidative stress, this should be an obvious choice for further studies.

4 | Discussion

Fertilization and its aftermath mark a critical period of epigenetic reprogramming in animal zygotes, setting the stage for embryonic development and cellular differentiation. As two of the most specialized cell types in the animal body unite to create a totipotent one, the gene expression landscape has to undergo a dramatic shift, with associated changes in chromatin structure and epigenetic marks (Tang and Hu 2024). In animals with external fertilization and rapid development, this reprogramming coincides with a series of extremely fast, synchronized cell divisions, where a specialized, shortened cell cycle consisting only of DNA synthesis (S phase) and mitosis (M phase) (Brantley and Di Talia 2024). In the absence of a proper growth phase and associated transcription of genomic transcripts, the cells of the embryos undergoing this process (also termed “cleavage”) become progressively smaller and rely solely on the presence of maternal factors for their function. However, after a species-specific number of cell divisions (13 cell cycles in *Drosophila*, 12 cycles in *Xenopus* and 9 cycles in zebrafish), the zygotic genome of the organism awakens, with concomitant elongation of the cell cycle. As the zygotic transcription in the embryo increases, a parallel destruction of maternal products can be also observed (Baroux et al. 2008; Jukam et al. 2017; Lee et al. 2014a; Vastenhouw et al. 2019).

The regulation and timing of this crucial phase, the MZT, has been the subject of numerous studies over the years, which have elucidated a number of key mechanisms that are instrumental in orchestrating this transition. N/C ratio, activation of cell cycle checkpoints and reorganization of chromatin are all important factors in the process (see above), but lately a string of studies have suggested that incorporation of certain non-canonical nucleotides into the genome might be also important for the process (Febrimarsa et al. 2023; He et al. 2019).

Traditionally only the presence of the epigenetically relevant 5mC and its derivatives (5hmC, 5acC and 5fC) has been considered “normal” in DNA, whereas the incorporation and/or presence of other noncanonical nucleobases, such as U or 6mA was seen as an error resulting from incorrect replication or mutagenic activity. As such, these errors had to be repaired as quickly as possible, and well characterized repair mechanisms, such as BER have been proposed to fulfill this function. Lately,

however, with the advent of more sophisticated analytic tools, closer examination revealed a dynamic genomic presence for some of these noncanonical nucleotides, and occasionally a patterned distribution in the early genome (Liu et al. 2016; Muha et al. 2012; Shu et al. 2018), raising the possibility of their direct role in regulating some aspects of the developmental process itself.

In our review we have investigated some of the noncanonical nucleobases in the genome that might affect the onset and progression of ZGA. While for some of these nucleobases the evidence is well established, for some is mostly correlative at this point. Yet, we think that current evidence warrants a closer look for the possible functional role of these nucleobases during ZGA.

5mC is fundamental in epigenetic regulation and plays a critical role in controlling cellular differentiation during development (Mattei et al. 2022; Smith and Meissner 2013; Zhu et al. 2018). Although a direct link between 5mC and ZGA has not been explicitly described, its broad genomic influence, which is conserved across vertebrate species, suggests that it likely contributes to ZGA in some capacity. Of note, in several teleost species the specific pattern of mCH observed around ZGA also suggests a role for 5mC in this process (Ross et al. 2020; Ross et al. 2023). Furthermore, for 5fC a direct role in the activation of tRNA genes during ZGA has been described in *Xenopus* and mice as well (Parasyraki et al. 2024).

The presence and developmental role of m^6A in maternal transcripts are well documented, yet the occurrence and putative role of its counterpart 6mA in the genomes of animals remains controversial (Boulias and Greer 2022; Gilbert et al. 2016; Kong et al. 2022; Meyer and Jaffrey 2017; Zaccara et al. 2019; Zhu et al. 2018). Besides being detected only in trace amounts in the genome, experimental evidence suggests that its presence primarily results from erroneous incorporation of derivatives from degrading maternal transcripts. Nevertheless, this does not imply that 6mA enrichment could not acquire a genomic function. This is corroborated by the finding that in *Hydractinia* embryos 6mA incorporation delays the onset of ZGA, likely due to interfering with transcription (Febrimarsa et al. 2023). Unlike in *Hydractinia*, 6mA in bilaterian embryos does not appear to be entirely random in distribution. For example, in *Drosophila*, the *zelda* gene is enriched in 6mA, where it may influence transcription factor binding, suggesting a more intricate regulatory function (He et al. 2019).

The presence of dUMP in genomic DNA is well described, but its direct link to ZGA has hitherto not been established. It is known that dTTP levels decline during cleavage stages (Liu et al. 2019). We hypothesize that if this decrease in dTTP levels is accompanied by an increase in the dUTP/dTTP ratio, then the rate of dUMP misincorporation likely increases, potentially influencing ZGA by recruiting DNA repair enzymes that modify chromatin accessibility. However, the potential role of a common uracil modification, 5hmU in this context remains unsubstantiated, as current literature does not establish a connection between 5hmU and ZGA.

8-oxoG is a spontaneously occurring but relatively frequent nucleobase modification associated with oxidative stress. Specific patterns of 8-oxoG distribution have been described, and in some cases, these patterns influence transcription factor binding or nuclear receptor function. However, it remains unclear whether this reflects a regulated process or merely the result of uneven oxidation and/or repair. The accumulation of 8-oxoG and its potential impact on ZGA has yet to be investigated.

The nonrandom genomic distribution of particular noncanonical nucleotides during early stages of development implies the presence of one or multiple processes that can result in such patterned distribution of modified nucleotides (Figure 5A). One possibility would be the targeted recruitment of particular modifier enzymes to certain genomic features which would localize their activity to those particular DNA stretches. An alternative explanation of the observed patterns could be that the enzymatic modification of the DNA occurs randomly, but selective repair processes will only remove the modifications in particular genomic contexts. Finally, high levels of noncanonical dNTPs in the cytoplasm also create opportunities for their random incorporation into the genome of dividing cells (Figure 2B). If the speed of repair processes is different in various subsets of genomic compartments, patterned distributions will be also observed.

Noncanonical nucleotides are usually removed by well characterized repair mechanisms, and their incorporation through replication has been traditionally considered to be erroneous, threatening genomic integrity. Recently, however, evidence emerged that the timely induction of DNA repair processes can be important for the accurate onset of genomic activation. For example, in early embryos of the cnidarian *H. symbiolongicarpus* the random introduction of 6mA into the genome triggers the activation of Alkbh1, which seems to be essential for the timely initiation of ZGA in this animal (Febrimarsa et al. 2023). Also, in *Drosophila*, zebrafish and *Xenopus* embryos the onset of ZGA and the concomitant lengthening of the cell cycles is associated with the acquisition (and presumably the activation) of Chk1, a known mediator of DNA damage response (Conn et al. 2004; Fogarty et al. 1997; Shimuta 2002; Zhang et al. 2014). But programmed DNA breaks have also been shown to have an important role in the activation of the germline genome in *C. elegans* (Wong et al. 2018).

Given the widespread distribution of some noncanonical nucleotides in the genome of early animal embryos and their dynamic removal during MZT, perhaps it is time to consider their putative role for ZGA (Figure 5B). Elevated levels of noncanonical dNTPs can arise either from the activity of *de novo* nucleotide synthesis or salvage pathways, or from recycling of ribonucleosides that originated from the programmed degradation of maternal transcripts (Figure 2B). These noncanonical dNTPs will be incorporated either randomly or directionally in the DNA of fast-proliferating cells present in the pre-MZT embryos and may serve as substrates for DNA repair processes that initiate around the onset of MZT. The active removal of noncanonical nucleotides, in turn, could contribute to the loosening and reorganization of the chromatin, which can be an important factor in the timely initiation of ZGA (Figure 5B). Indeed, DNA breaks are not only important for the activation of the germline genome in worms, but the activation

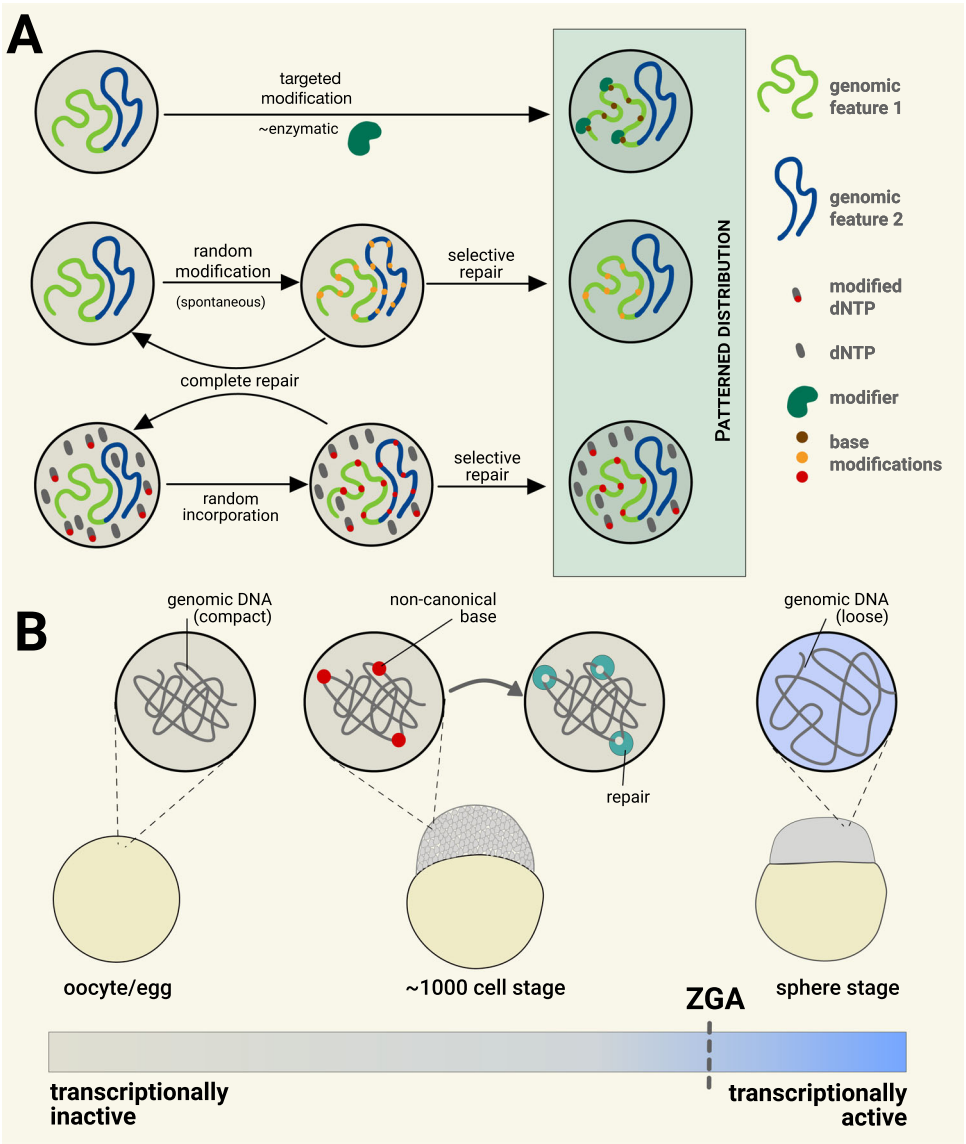

FIGURE 5 | The origin of nonrandom genomic patterns of noncanonical nucleotides and a possible role for such nucleotides in ZGA. (A) A patterned distribution of noncanonical nucleotides could arise as the result of targeted modification of specific genomic regions by modifying enzymes, or random modifications, followed by selective repair, or by the random incorporation of noncanonical nucleotides from the cytoplasmic pool, followed by selective repair processes. (B) Incorporation of noncanonical nucleotides (red dots) into the compact genome during cleavage is followed by their removal before the onset of ZGA. The BER process at this stage can leads the appearance of DNA breaks. These could facilitate the decompaction of the genome during ZGA, as shown here through the example of a zebrafish embryo.

of DNA damage pathways results in the reorganization of the chromatin more generally (Santos et al. 2020; Wong et al. 2018).

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