Title: Reversible histone acetylation during preimplantation embryo development in mammals

Authors: Giulia Musmeci, Fernanda Fagali Franchi, Francesca Mossa, Alberto Maria Luciano, Valentina Lodde, Federica Franciosi

Giulia Musmeci, ORCID ID: 0000-0001-8099-2836
Reproductive and Developmental Biology Laboratory, Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi (LO), Italy

Fernanda Fagali Franchi, ORCID ID: 0000-0002-9859-0576
Reproductive and Developmental Biology Laboratory, Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi (LO), Italy

Francesca Mossa, ORCID ID: 0000-0001-5517-1307
Department of Veterinary Medicine, University of Sassari, Sassari (SS), Italy

Alberto Maria Luciano, ORCID ID: 0000-0002-8016-9919
Reproductive and Developmental Biology Laboratory, Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi (LO), Italy

Valentina Lodde, ORCID ID: 0000-0002-9768-2292
Reproductive and Developmental Biology Laboratory, Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi (LO), Italy

Federica Franciosi, ORCID ID: 0000-0001-8742-0291
Reproductive and Developmental Biology Laboratory, Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi (LO), Italy

Corresponding Author: Federica Franciosi
Reproductive and Developmental Biology Laboratory, Department of Veterinary Medicine and Animal Sciences, University of Milan
Via dell'Università, 6 - 26900
Lodi (LO) – Italy
federica.franciosi1@unimi.it
+39 02503 34364

Abstract

Histone acetylation is an epigenetic modification responsible for changes in chromatin architecture, accessibility, and ultimately gene expression. At the onset of a new life, when the fully differentiated parental genomes fuse together to generate a new totipotent cell, the gametes' epigenetic program must be erased, and new ones are progressively installed. Together with other epigenetic modifications, histone acetylation participates in the early events of embryogenesis, undergoing dynamic changes that involve several amino acid residues on different histone proteins. By analyzing studies that followed these changes during the preimplantation development in different mammals, we identified critical windows of acetylation/deacetylation in relation to the oocyte to zygote transition, the activation of the embryonic genome, and the specification of cell lineages, all crucial events for early embryo development, the establishment of pluripotent embryonic tissue, and ultimately of a multicellular organism.

Finally, this survey points out the possibility that while contributing to the necessary plasticity of the embryonic stem cells, the reversibility of histone acetylation/deacetylation patterns renders this mechanism prone to be hijacked by environmental conditions, such as maternal diet or pollutants, leading to the alterations of epigenetic marks that can be potentially transmitted to the daughter cells and up to adulthood.

Keywords

Oocyte, zygote, blastocyst, epigenetics, embryo metabolism, histone H3, histone H4, fetal programming of adult disease

Reversible histone acetylation during preimplantation embryo development in mammals

Multicellular organisms originate from a single totipotent cell, the zygote, that in turn derives from fully differentiated gametes. Therefore, the maternal and paternal genomes contributing to the zygote must undergo complete nuclear and epigenetic reprogramming to establish a totipotent state and progressively install the required changes. This program of 'molecular erasure and writing' ultimately drives the differentiation of the pluripotent embryonic tissues.

Histone acetylation, like many other epigenetic modifications, endures drastic remodeling, both at the global and site-specific level, which are necessary to modify the chromatin conformation and accessibility, hence controlling the ability to interact with transcription factors and, consequently, having a role in gene expression (Shirvaliloo, 2022).

The reversible nature of histone acetylation, regulated by enzymes that introduce or remove the acetylated groups from specific amino acid residues (Lodde, Luciano, Franciosi, Labrecque, & Sirard, 2017; Peserico & Simone, 2011), is particularly evident at the oocyte-to-zygote transition, entailing the removal of the gamete-specific marks. However, reports of changes in the histone acetylation patterns also exist for later crucial developmental passages, as the embryo genome activation (EGA) and the first cell lineages commitments, indicating the participation of histone acetylation/deacetylation events in the progressive fine tuning of the functions of the embryonic genome.

However, this exact reversible nature also exposes the program of histone acetylation to the influence of external factors that, by acting on the availability of substrates and/or on the activity of the enzymes, might interfere with the normal patterns of histone acetylation, affecting the survival of the embryo, the dynamic of development, or even setting the stage for future anomalies, as postulated by the hypothesis of developmental programming of adult disease.

After a brief recapitulation of the main events in mammalian embryo development, with this review we will analyze the key histone acetylation changes of the prominent amino acid residues during preimplantation development and finally explore the scenarios that may compromise their establishment.

Most of the knowledge on histone acetylation during early embryo development comes from studies on humans, mice, pigs, and cattle, while research on other mammals, such as dromedaries, rabbits, buffalos, and primates, is relatively rare. This review will emphasize findings from the primary species of interest. Furthermore, due to the capacity of the embryo to develop outside the maternal body for several days, many of the studies considered rely on experimental models of *in vitro* embryo culture.

1. Main events of preimplantation embryo development in mammals

The early stages of embryo development comprehend a series of events starting immediately after fertilization and resulting in the formation of a multicellular organism. This process is

crucial for successful implantation and subsequent fetal growth (Alberio, 2020). After fertilization, the zygote is formed through the fusion of the male and female pronuclei, resulting in a single cell that contains a complete set of chromosomes inherited from both parents. The zygote then undergoes a series of mitotic divisions, with the initial stages being regulated by stored mRNAs and proteins of maternal origin before a switch to embryo-encoded transcripts is made. In mammals, the early divisions lead to the progression from the 2-cell stage to a more complex structure, the morula, which typically consists of around 32-64 blastomeres. These first cellular divisions mark a critical phase in the embryo's development as they anticipate and prepare the cellular and molecular machinery for successive differentiation and specialization. Upon reaching the morula stage, the embryo begins to form a fluid-filled cavity called the blastocoel. This event marks the differentiation of the first two distinct cell populations: the inner cell mass (ICM), which will give rise to the fetus, and the trophectoderm (TE), which will form the embryonic annexes. The ICM will further differentiate into an epithelial monolayer named primitive endoderm (PrE) or hypoblast, which contributes to the yolk sac and the remaining enclosed epiblast cells (Epi), which give rise to all the fetal tissues (Cui & Mager, 2018; Rossant, 2018; Wilkinson, Zorzan, & Rugg-Gunn, 2023). At the beginning of blastocoel formation, the early blastocyst remains enveloped within the zona pellucida, an oocyte-derived glycoprotein shell. Later, with the enlargement of the cavity and continuous cell proliferation, the blastocyst expands and the zona pellucida thins, leading to embryo hatching.

Once free, the hatched blastocyst continues its expansion, increasing in size. In species such as cattle and pigs, this phase involves flattening out and transitioning to an elongated shape, significantly increasing its surface area and contact with the maternal endometrium (Davenport, Ortega, Johnson, Seo, & Spencer, 2023; Miles, Walsh, Rempel, & Pannier, 2023). This elongation and flattening are typical of non-invasive implantation, where the increased surface contact with the uterine lining facilitates nutrient exchange and placental development. In contrast, in mice and humans implantation is invasive and the blastocyst does not elongate before implantation, but it keeps a cylindrical or spherical shape (Kagawa et al., 2022; Weberling & Zernicka-Goetz, 2021).

The preimplantation stages of development are characterized by cell proliferation and differentiation, setting the stage for successful implantation and the subsequent development of the embryo within the maternal environment (Zhang, Yang, & Wu, 2007). Various factors, including hormonal levels, uterine receptivity, and the health and quality of the embryo itself influence the success of early embryogenesis. If implantation is successful, the embryo begins to establish a connection with the maternal blood supply, ensuring proper nourishment and support for subsequent development.

The preimplantation stages of embryogenesis can be replicated *in vitro* using standardized protocols, except for the stage of blastocyst elongation, which is still experimental (Ramos-Ibeas et al., 2020). Thanks to *in vitro* embryo production (IVP) protocols, embryos of several mammalian species are cultured in specialized media for up to 7-8 days until the blastocyst

stage. Bovine IVP is one of the best established protocols, allowing to obtain 20 to 40% blastocyst development starting from prophase I-arrested, fully-grown immature oocytes (Lonergan, Rizos, Ward, & Boland, 2001). Upon isolation from the follicular environment, such oocytes resume meiosis and reach metaphase II (MII), corresponding to the stage at which they are when ovulation occurs. After this step, called *in vitro* maturation (IVM), they can be fertilized *in vitro* (IVF), and the resulting zygotes are cultured for several days. When reaching the morula-early blastocyst, the embryos can be transferred into recipients or cryopreserved for future, timed transfer. However, only high-quality embryos can be successfully used for cryopreservation, as they bear the potential for higher pregnancy rates (Layek et al., 2022).

During the early stages of development, the embryo utilizes stored maternal mRNAs and proteins before the embryonic genome becomes activated. The EGA occurs with species-specific timing: around the 4-8-cell stage in humans, the 8-16-cell stage in cattle, the 4-cell stage in pigs, and the 2-cell stage in mice (L. Li, Lu, & Dean, 2013), with large mammals exhibiting comparable timelines during the preimplantation stages up to the hatching, compared to mice. Similarly, after fertilization, human embryos progress to the blastocyst stage in approximately 7 days, as cows. Conversely, the development of murine embryos is faster, and the blastocyst stage is already reached at 4 days after fertilization. Indeed, in mice, compaction begins earlier, at the 8-16 cell stage, and blastocoel cavity formation occurs between the 16-32 cells (Kidder & Watson, 2005). In contrast, bovine embryos compact near the 32-cell stage, with cavitation generally observed after the 64 cells (Van Soom et al., 1997).

Besides the timeline, the mechanisms regulating early cell lineage specification in the mouse differ from those of large mammals, as evidenced by gene expression profiling (Berg et al., 2011; Blakeley et al., 2015; Fogarty et al., 2017; Gerri et al., 2020; Niakan & Eggan, 2013). In mice, the expression of genes such as the POU domain, class 5, transcription factor 1 (*Pou5f1*, also known as octamer-binding transcription factor 4 - *Oct4*), and SRY-box 2 (*Sox2*) is critical for the maintenance of the pluripotency in the undifferentiated cells of the ICM. At the same time, caudal type homeobox 2 (*Cdx2*) promotes the differentiation of the TE by repressing the expression of the pluripotency factors (Kim, Chu, Shen, Wang, & Orkin, 2008; Niwa et al., 2005). The ICM is further separated in Epi and PrE, in which the Epi marker, homeobox protein NANOG (*Nanog*), and the PrE marker, GATA-binding factor 6 (*Gata6*), repress each other during the second lineage segregation (Thompson et al., 2022).

In higher-order mammals, the expression of *POU5F1* is not limited to the ICM as in mice, but it is widespread, extending to all cells in the Day 8 blastocyst (Aguila, Osycka-Salut, Treulen, & Felmer, 2022; Fogarty et al., 2017), while *SOX2* is detected in the ICM nuclei of bovine, mouse, and human embryos (Aguila et al., 2022; De Paepe et al., 2013; Yao, Zhang, & Shuai, 2019). Overall, the transcription factors *POU5F1* and *CDX2* exhibit comparable temporal and spatial expression patterns in human and bovine embryos (Berg et al., 2011; A. E. Chen et al., 2009; Niakan & Eggan, 2013), while mice display notable differences from these species since the proteins POU5F1 and CDX2 start to be detected from the 8- and the 16-cell stages, respectively

(Niakan & Eggan, 2013; Niwa et al., 2005). Overall, both bovine and murine models offer distinct advantages and limitations for studying early embryonic development. The features of each model highlight the necessity for a complementary approach to enhance the ability to investigate biological processes and refine the strategies to study human development (Carreiro, Dos Santos, Luedke, & Goissis, 2021).

1.1 Epigenetic remodeling in preimplantation embryo development

As anticipated above, during the early stages of embryogenesis, the embryo undergoes significant epigenetic remodeling. Epigenetic modifications are heritable changes that alter chromatin structure and gene expression without modifying the DNA sequence. Thanks to these mechanisms, a single genotype expresses multiple phenotypes, such as the cells composing different tissues and organs in an organism or during the commitment and determination of cell fate. They can be passed down to daughter cells and even from parents to offspring through multiple generations via transgenerational epigenetic inheritance (Moelling, 2024). Epigenetic changes include an array of different mechanisms, such as DNA methylation at specific cytosine residues, covalent modifications of histone proteins, and expression of non-coding RNAs (ncRNAs) (Kiefer, 2007; Mondal & Kanduri, 2013).

The embryo epigenetic reprogramming begins after fertilization. For instance, the DNA is globally demethylated and DNA methylation is then re-established according to sex-specific patterns, with the notable exception of imprinted genes which retain their methylation status throughout these processes (Y. Chen, Wang, Guo, Dai, & Zhang, 2023). Genomic imprinting is an epigenetic process that leads to monoallelic expression based on parental origin (Crespi, 2019). Imprinted genes are crucial in mediating fetal and childhood growth, significantly influencing early developmental patterns and metabolic programming. Disruptions in imprinting can result in abnormal phenotypes linked to human diseases, such as Prader–Willi syndrome, an epigenetic variation at the H19 gene, which encodes an imprinted maternally expressed non-coding transcript, and at insulin-like growth factor 2 (*Igf2*), both of which influence birth weight and long-term health risks like obesity and type 2 diabetes (Peters, 2014).

In the embryo, epigenetic mechanisms ensure that a single genotype – the one formed by the syngamy of paternal and maternal genomes in the zygote – is progressively committed to express different phenotypes, therefore allowing proper development, influencing processes such as lineage commitment, blastocyst formation, and implantation.

However, epigenetic patterns can be altered by environmental factors, such as nutrition, stress, and pollutants, during critical periods of development (Peral-Sanchez, Hojeij, Ojeda, Steegers-Theunissen, & Willaime-Morawek, 2021). Consequently, epigenetic modifications are considered key mechanisms through which early-life environmental exposures impact long-term health outcomes. Recent research has provided valuable insights into how specific environmental exposures can affect epigenetic mechanisms and predispose individuals to various chronic diseases. For example, maternal consumption of different nutrients during the first trimester of pregnancy has been associated with epigenetic modifications that alter gene

expression and may result in increased susceptibility to developing obesity and related metabolic changes, as proposed by the Developmental Origins of Health and Disease (DOHaD) (Barker, 2007), also known as fetal programming of adult disease. This theory highlights how environmental conditions, like maternal malnutrition, during early development can influence susceptibility to chronic diseases in adulthood (Bokor et al., 2024). Similarly, exposure to stress or toxicants during critical periods of development can have significant consequences on epigenetic mechanisms and increase the risk for altered physiological and psychiatric outcomes in the offspring (de Magalhães-Barbosa, Prata-Barbosa, & da Cunha, 2022; Dieckmann & Czamara, 2024). Despite the intensive research on this topic, the critical window(s) of exposure is not entirely defined, and a possible involvement of the first stages of embryo development has not received full attention. Furthermore, DNA methylation has been mainly considered thus far. However, the interplay of various epigenetic mechanisms is likely to occur.

In the next session we will describe the dynamics of histone acetylation during the normal preimplantation embryo development in the main studied mammalian species and examine the putative influence that the environment can exert on such dynamics.

2. Histone acetylation changes during early embryo development

The amino-terminal tails of histone proteins extend outward from the nucleosome and undergo various post-translational covalent modifications, including acetylation, methylation, phosphorylation, biotinylation, sumoylation, crotonylation, and lactylation (Gao et al., 2024; Jenuwein & Allis, 2001; Strahl & Allis, 2000; Yang et al., 2024). These modifications influence chromatin architecture and DNA accessibility, affecting transcriptional activity and representing important epigenetic mechanisms for regulating gene expression.

Histone acetylation is one of the better-understood modifications and it is mediated by histone acetyltransferases (HATs), which add acetyl groups, and histone deacetylases (HDACs), which remove them. Acetylation occurs predominantly on lysine residues (K) within the N-terminal tail, neutralizing their positive charge and weakening the interaction between the histone proteins and the negatively charged DNA (Bannister & Kouzarides, 2011; Millán-Zambrano, Burton, Bannister, & Schneider, 2022). Importantly, histone acetylation directly increases the rate of transcription *in vitro* (Durrin, Mann, Kayne, & Grunstein, 1991; Protacio, Li, Lowary, & Widom, 2000). Histone acetylation occurs on several core histones, each contributing uniquely to chromatin regulation and gene expression.

2.1 Histone H3

Histone H3 is the most studied and significant target for acetylation, particularly at the K residues H3K9, H3K14, and H3K27, which play essential roles in transcriptional regulation and chromatin structure. Additional acetylation sites, including H3K18, H3K23, H3K56, H3K64, and H3K122, have been noted, although they have been less extensively investigated. A graphical

representation of the acetylation dynamics of key residues on histone H3 during early embryo development is provided in Figure 1.

The acetylation of **lysine 9** on histone H3 (H3K9ac) has been a focal point in early embryonic development studies. Research in mice using immunofluorescence or Chip-seq techniques has not demonstrated significant fluctuations in H3K9 acetylation levels during early embryogenesis up to the blastocyst stage (Bošković et al., 2012; Guo et al., 2012; J. Li et al., 2022; Stein, Worrad, Belyaev, Turner, & Schultz, 1997).

The bovine model appears to follow a similar pattern, as observed in some immunofluorescence studies (Bošković et al., 2012; Salehi, Abouhamzeh, Hosseini, Zare, & Bakhtari, 2020; M. Wang et al., 2017). However, other studies using the same technique have reported a reduction in H3K9ac levels at the 8-cell stage (X. Wu, Hu, Wang, Li, & Yu, 2020) (X. Wu et al., 2011), which coincides with the EGA in cattle. This observation suggests a putative role of this residue in epigenetic reprogramming at this critical stage. Notably, the same study also considered H3K9ac starting from the oocytes, observing a decrease in acetylation at the MII stage, prior to fertilization, followed by a rapid increase post-fertilization, pointing out that H3K9ac is erased after meiosis resumption (X. Wu et al., 2020). A similar pattern was also reported for H3K18ac in the same study (X. Wu et al., 2020).

Only one study has investigated the 4-cell stage in porcine embryos, which corresponds to the EGA in pigs. This study reported a reduction in H3K9ac levels, similar to what was observed in cattle oocytes. However, conclusions should be drawn carefully since the antibody used also stains H3K14ac, implying that the observed signal may overlap with the detection of other residues (Nguyen, Lo, Chuang, Jian, & Ju, 2011).

Research on histone H3 **lysine 14** acetylation (H3K14ac) has revealed fluorescence signal reduction at the 4-cell stage in porcine embryos, coinciding with the EGA (Liu et al., 2012; Sun et al., 2020), such as observed for H3K9 in the cattle, again suggesting a dynamic epigenetic modulation specific to this developmental transition. The observation of high global histone acetylation levels before the EGA, at a time of transcriptional silencing, could be reconducted to this modification's multifaceted functions. Indeed, histone acetylation not only facilitates gene transcription by promoting the accessibility of genetic information to transcription factors, but it also has a significant role in regulating chromatin structure (Shahbazian & Grunstein, 2007). An increase in the fluorescent signal was then detected at the blastocyst stage in porcine oocytes (Liu et al., 2012; Nguyen et al., 2011; Sun et al., 2020).

This residue doesn't reveal significant fluctuations in studies conducted on mouse and bovine embryos (Carey et al., 2015; Stein et al., 1997; P. Zhao et al., 2019). In contrast, a study in mice examining stages from the 2-cell to the blastocyst reported a substantially stronger acetylation signal at the 2-cell stage that slightly decreased thereafter and was stably maintained up to the blastocyst stage (Guo et al., 2012). Unfortunately, many studies have focused on a single developmental stage, failing to highlight dynamic changes that might occur

throughout the preimplantation period. Further investigation is therefore required to resolve these discrepancies and better understand the regulation of H3K14ac during early development.

Patterns of acetylation of **lysine 27** (H3K27ac) differ across the same species. In fact, in mice, conflicting findings have been reported: one study which used ChIP-seq technique observed reduced acetylation at the 8-cell stage (K. Wu et al., 2023), while another study, also using ChIP-seq, reported a decrease as early as the 2-cell stage (J. Li et al., 2022), corresponding to EGA in the mice. A yet additional study using μChIP-seq reported a marked increase in the genomic coverage of H3K27ac from oocytes to the 2-cell stage, but no significant changes were observed between the 2-cell and 8-cell stages (Dahl et al., 2016). Conversely, a different study using immunofluorescence revealed a dramatic decrease in H3K27ac levels from the prophase I stage to the MII in mice (M. Wang, Chen, & Zhang, 2022). These levels were rapidly restored in zygotes to values comparable to those observed in prophase I oocytes, suggesting that de novo acetylation occurs shortly after fertilization. Notably, acetylation levels decreased again at the early 2-cell stage. These findings were further corroborated using the CUT&RUN technique, which demonstrated noncanonical broad H3K27ac domains in prophase I oocytes and in zygotes, indicating chromatin hyperacetylation during these stages (M. Wang et al., 2022). Notably, in a study in humans using ChIP-seq on patients undergoing intracytoplasmic sperm injection (ICSI) to overcome infertility, genomic regions marked by broad H3K27ac domains are narrowed down during development. At the 8-cell stage and onward, most of the broad H3K27ac domains (> 10 kb) detected in 2-cell embryos disappear or transit to narrow H3K27ac peaks (K. Wu et al., 2023).

A recent study (Zhou, Halstead, Bonnet-Garnier, Schultz, & Ross, 2023) reported increased H3K27ac levels in cattle from the morula stage onward, with pronounced acetylation in both the ICM and TE at the blastocyst stage, suggesting its role in lineage specification.

Other lysine residues, such as H3K18, H3K23, H3K56, H3K64, and H3K122, remain understudied, with limited and even more inconsistent data reported across species and developmental stages. An immunofluorescence signal of histone H3 acetylation at **lysine 18** (H3K18ac) was erased after re-entry into the meiotic cell cycle in bovine oocytes and then newly established in the zygotes. Then, it significantly decreased at the 8-cell stage, suggesting a transient reduction in acetylation during EGA (X. Wu et al., 2020; X. Wu et al., 2011). This trend reverses by the morula stage, with a significant increase in staining intensity. Interestingly, at the blastocyst stage, H3K18ac displays different intensities according to the localization, with the acetylation signal being less intense in the ICM compared to the TE cells (X. Wu et al., 2011). This difference likely reflects the contrasting states of these two lineages: the TE is already differentiated and committed to forming the extraembryonic structures, whereas the ICM remains in a pluripotent state, capable of giving rise to all embryonic tissues. While these observations suggest a potential link between H3K18ac and lineage specification, the

underlying mechanisms and functional implications remain unclear. A similar pattern has also been observed for some residues of histone H4, as reported below.

The acetylation of **lysine 23** on histone H3 (H3K23ac) has shown contrasting patterns in different species, reflecting variability in the epigenetic regulation during preimplantation development. In mouse embryos, H3K23ac was observed to be distributed uniformly throughout the nucleus, with no apparent stage-specific changes (Stein et al., 1997). Conversely, H3K23ac is absent in porcine embryos during the 1-, 2-, 4-, and 8-cell stages but becomes enriched at the blastocyst stage (Lin et al., 2020). These discrepancies highlight potential species-specific regulatory roles for H3K23ac in embryogenesis. Notably, its enrichment at the blastocyst stage in porcine embryos suggests a potential link to the emergence of differentiated lineages. Still, in this case, more studies are needed to clarify its function and relevance to lineage-specific processes.

The acetylation patterns of **lysine 56, 64, and 122** on histone H3 (H3K56ac, H3K64ac, and H3K122ac) remain poorly characterized, with limited studies reporting conflicting findings. In a mouse model, H3K56ac and H3K64ac were consistently present throughout preimplantation, whereas H3K122ac, although detected from prophase I to 8-cell stage, was observed at low levels before implantation. These findings suggest a more prominent role for H3K56ac and H3K64ac than H3K122ac in mouse preimplantation development (Ziegler-Birling, Daujat, Schneider, & Torres-Padilla, 2016). In contrast, a study in human embryos demonstrated that all three modifications are detectable throughout the preimplantation stages, albeit at varying levels (X. F. Wang, Xie, Guo, Su, & Zhou, 2020). H3K64ac showed relatively stronger signals, while H3K122ac levels were much weaker. Interestingly, H3K56ac exhibited a peak at the 2-cell stage. These interspecies differences underscore the need for standardized methodologies to elucidate the specific roles of these acetylation marks.

2.2 Histone H4

Histone H4 acetylation at **lysine 5, 8, 12, and 16** (H4K5ac, H4K8ac, H4K12ac, and H4K16ac) plays a crucial role in chromatin remodeling and transcriptional regulation during the oocyte to zygote transition and the early embryo development. Despite their importance, studies on these residues remain limited, with findings revealing complex and stage-specific patterns influenced by environmental stimuli and experimental conditions, similar to those emerged from the survey of H3 acetylation patterns. A graphical representation of acetylation dynamics at key residues on histone H4 during early embryo development is provided in Figure 2.

A study on bovine embryos has shown similar dynamics for H4K5ac and H4K8ac during early development (X. Wu et al., 2020). Specifically, the fluorescent signal of H4K5ac and H4K8ac is erased after meiosis resumption and during fertilization, while they are re-established on the male and female chromatin at 4 and 8 hrs postfertilization, respectively (Ma & Schultz, 2008; X.

Wu et al., 2020). Notably, in the same study, histone methylations exhibit no significant changes during meiosis. As preimplantation development progresses, the signal of H4K5ac and H4K8ac increases significantly after the morula stage (X. Wu et al., 2011), as observed in another study (X. Wu et al., 2020). An independent study has also confirmed the deacetylation of MII bovine oocytes (Lodde et al., 2021).

In mice, a decrease in the H4K5ac fluorescence signal was observed in MII oocytes, followed by a re-establishment in the zygote and a gradual decrease at the 8-cell and morula stages, followed by a resurgence at the blastocyst stage (Ma & Schultz, 2008). The H4K5ac and H4K8ac levels of the blastomeres during interphase seem to be more pronounced at the nuclear periphery from the 2-cell to the 8-cell stages (Stein et al., 1997; X. Wu et al., 2020). Interestingly, during mitosis, the H4K8ac signal becomes particularly intense at the chromosome periphery (X. Wu et al., 2020). These observations highlight the potential regulatory roles of H4K5ac and H4K8ac in key transitions, such as EGA and chromatin organization. However, the specific functional implications of their localization at the nuclear and chromosome peripheries remain to be elucidated.

A study conducted in 2007 on mouse embryos reported a homogeneous immunofluorescence staining pattern of acetylated H4 (AcH4) from the zygote to the blastocyst stage in both *in vivo* and IVF-derived embryos (J. C. Huang et al., 2007). Conversely, in 2004, another study that included the oocytes' prophase I and MII stages described distinct patterns of hyperacetylated H4 (Sarmento et al., 2004). In immature prophase I oocytes, hyperacetylation was confined to condensing chromatin surrounding the nucleolar-like body, while at metaphase, global levels of acetylation dramatically declined, with almost no staining on egg chromatin. However, strong AcH4 signals were observed in interphase blastomeres. At the blastocyst stage, AcH4 staining was less intense in the ICM compared to the TE. We observed similar patterns in bovine blastocysts obtained by IVP and immunostained with antibodies directed against the acetylated forms of H4K8 and, to a lower extent, H4K12 (Figure 3, unpublished data). The difference in acetylated signal intensity may reflect the commitment of the TE toward a differentiated state compared to the ICM's pluripotent state (Sarmento et al., 2004).

Notably, the residues seem particularly sensitive to environmental stimuli. In mouse zygotes, the signals of H4K8ac, H4K12ac, and H4K16ac are significantly reduced three hours after somatic cell nuclear transfer (SCNT) compared to three hours after intracytoplasmic sperm injection (ICSI), where acetylation levels are higher (F. Wang, Kou, Zhang, & Gao, 2007). This discrepancy may arise from different requirements between somatic cells, bearing histones and the spermatozoa, bearing protamines, to reorganize the nucleosomes. Additionally, it may suggest that external factors, such as the fertilization mode, influence these residues' acetylation patterns. In line with this hypothesis, we repeatedly observed that the oocytes' acetylation at specific residues is affected by the mode of maturation. Indeed, acetylation at the H4K16 is retained in *in vivo* ovulated horse oocytes, but it becomes undetectable in *in vitro* matured ones (Franciosi, Goudet, et al., 2017; Franciosi, Tessaro, et al., 2017). Notably, other

H4 residues, K8 and K12, were not affected (Franciosi et al., 2012), probably indicating a different sensibility of residue-specific histone acetyl-transferases/deacetylases to environmental conditions.

In porcine embryos, at the time of EGA, which occurs at the 4-cell stage, a marked reduction in the H4K8ac signal has been observed (Cervera, Martí-Gutiérrez, Escorihuela, Moreno, & Stojkovic, 2009). However, this acetylation level recovers at the blastocyst stage, aligning with developmental requirements for chromatin remodeling and lineage specification.

Studies on H4K12ac and H4K16ac conducted in mouse, bovine, and porcine embryos did not identify significant differences at key developmental stages (Cao et al., 2019; J. Huang et al., 2016; Stein et al., 1997; Xiao et al., 2022). Interestingly, an increase in H4K16ac fluorescence was only observed in one study conducted in the porcine blastocyst (Cao et al., 2017). Nonetheless, the limited focus on these residues highlights the need for more comprehensive research to uncover their potential regulatory roles during embryogenesis.

Interestingly, among the lysine residues of histone 4, K 5 and K8 emerge as the most dynamically regulated and consistently implicated in acetylation patterning during early embryo development. Their pronounced stage-specific acetylation patterns, particularly during critical transitions, such as the MII stage and EGA, suggest a central role in orchestrating chromatin remodeling and transcriptional activation. However, future studies are essential to clarify whether the observed acetylation dynamics reflect universal mechanisms or species-specific variations and elucidate the interplay between different acetylation marks in shaping the embryonic epigenetic landscape.

2.3 Histones H2A and H2B

While much attention has been given to histones H3 and H4 modifications, fewer studies have focused on histone H2A and H2B during early embryonic development. Among the limited studies available, an early investigation conducted in 1997 on mouse embryos revealed that histone H2B was uniformly distributed in the nuclei at the 2-cell stage. In contrast, the single acetylated form of H2A (H2A.Ac5) localization showed enrichment at the nuclear periphery in 2-cell embryos (Stein et al., 1997).

More recently, another study compared the dynamics of H2A.Z acetylation (H2A.Zac) in bovine and mouse embryos, revealing differences between these species (Bošković et al., 2012). In mice, H2A.Zac was undetectable in embryonic chromatin at the early 2-cell stage and began to re-incorporate it at very low levels only in the late 2-cell stage. In contrast, in bovine embryos, H2A.Zac was strongly enriched on the maternal chromatin of zygotes and subsequently exhibited a uniform distribution across all nuclei during later stages.

These findings suggest that the dynamics of H2A.Z acetylation may not be conserved across mammals and may reflect species-specific regulatory mechanisms during early development. However, the scarcity of studies on H2A and H2B acetylation underscores the need for further research to elucidate their precise roles in chromatin remodeling and EGA.

3. Reversible histone acetylation/deacetylation and the adaptation to environmental conditions

During preimplantation embryo development, cells use the energetic substrates required for cell division, growth, and differentiation that are provided through the maternal reproductive organs (ovarian follicle for the oocyte, oviduct and uterus for the embryo) or in the culture medium. The embryo metabolism is an intricate and dynamic process that varies according to the stages of oocyte/embryo growth and it is crucial for successful development (Bruna de Lima, Cristina Dos Santos, & Sirard, 2023). For instance, during EGA, the embryo switches from anaerobic to aerobic metabolism, consequently changing substrate requirement for energy production (Leese, 1995; Martin, 2000).

A recapitulation of key biochemistry concepts is provided to better understand the mechanisms behind embryo metabolism and how the maternal/cultural environment may affect it.

3.1 Embryo metabolism

Glycolysis breaks down glucose to produce 2 molecules of ATP, pyruvate and lactate. Pyruvate produced during glycolysis can be oxidized into acetyl-CoA and enter the tricarboxylic cycle (TCA), also known as the citric acid or Krebs cycle. The TCA cycle is a mitochondrial pathway essential for energy production and biosynthetic processes, also during embryonic development (Chakrabarty & Chandel, 2022; Sharpley, Chi, Hoeve, & Banerjee, 2021). It takes place in the mitochondrial matrix and comprises 8 enzymes, except the outlier succinate dehydrogenase, which is related to the respiratory chain on the inner mitochondrial membrane. The TCA also plays a role in replenishing precursors for the storage form of fuels such as amino acids and cholesterol (Cavalcanti et al., 2014). The TCA cycle does not require oxygen directly; however, oxygen is needed for the final phase of aerobic respiration, the oxidative phosphorylation, which produces most of the ATP. The presence of coenzymes like NAD+ and FAD controls and regulates the Krebs cycle, while high concentrations of NADH inhibit it (Alabduladhem & Bordoni, 2024).

Before entering the TCA cycle, organic molecules, like carbohydrates, lipids, and amino acids, are transformed directly or through a further transformation in acetyl-CoA, a molecule formed by an acetyl group (CH3CO-) and an acyl transporter called coenzyme A. The acetyl group is then oxidized, and the energy obtained is stored as ATP in association with oxidative phosphorylation. However, the primary source of acetyl-CoA remains glycolysis. In conclusion, the TCA cycle integrates glucose, pyruvate, lactate, and amino acids into a cohesive energy production system vital for embryonic development (Figure 4). Its ability to maintain energy homeostasis and provide biosynthetic precursors is essential for the rapid proliferation and differentiation of embryonic cells.

During the first cleavages up to the morula, the embryo uses mainly pyruvate and partially lactate to generate energy in most species, including humans (Lee & Rinaudo, 2024).

Glycolysis is also present during these stages, but less active (Lee & Rinaudo, 2024). Initially the energy requirements are low, and the metabolism is mainly sustained by transcripts and proteins produced and stored during oocyte growth and maturation. At this point, the high ATP:ADP ratio maintains the inhibition of phosphofructokinase, limiting glucose oxidation. As the embryo develops, glycolysis increases, and glucose is transported into the blastomeres by the facilitated glucose transporters GLUT1 and GLUT3 (de Lima et al., 2020; Pantaleon, Harvey, Pascoe, James, & Kaye, 1997). Hence, as the embryo reaches the blastocyst stage, the glucose becomes the dominant substrate, most likely to face the energy demands of the Na+/K+ ATPase pump necessary to form the blastocoel cavity (Barrie, 2020; Leese et al., 2016). In addition to the increase in glycolysis, there is also an increase in oxygen consumption. In fact, the TE relies on oxidative phosphorylation, while ICM remains glycolytic in mammals (Lee & Rinaudo, 2024).

3.2. Environmental conditions and histone acetylation

The embryo metabolism is highly sensitive to environmental changes, which makes bioenergetics monitoring challenging but vital for facing physiological and pathological conditions and allows the adaptation and survival of the newly formed individual. Environmental variability can be brought about by changes in nutrient availability (bulk or specific components), oxygen concentration, and redox state (Gardner & Harvey, 2015). When one resource is scarce, cells redirect to alternative metabolic pathways (J. Zhao et al., 2021). Maintaining this balance is critical for early development, and adaptation mechanisms - including the regulation of reactive oxygen species and mitochondrial function - play an important role (Chakrabarty & Chandel, 2022; Harvey, 2019). In this sense, to better explain the correct metabolic balance, Leese et al. (Leese et al., 2016) introduced the concept of a 'Goldilocks zone', describing the optimal range of metabolic activity required for the best developmental outcomes, implying that too little and too much are not ideal, while what is correct in the end is the right amount.

Beyond the metabolic adaptation, it becomes intriguing to investigate the complex relationship between metabolism and molecular regulation. In this sense, the term “metaboloepigenetics” (Donohoe & Bultman, 2012) has been coined to define the interaction between energy metabolism, epigenetics, and molecular control mechanisms. As anticipated, in reproductive sciences, metabolic demands change at different developmental stages, particularly between cleavage and blastocyst formation, and these requirements are species-specific (Krisher et al., 2015; Milazzotto, Noonan, & de Almeida Monteiro Melo Ferraz, 2022). Preimplantation embryos rely on metabolic plasticity to survive in varying conditions, as they are constantly exposed to fluctuating environmental factors and/or nutrient availability. It is, therefore, conceivable that changes in energetic supplies, through the maternal diet or the culture medium, determine alterations of the ideal TCA substrate/product concentration that, in turn, can be ‘sensed’ by the cell and used to install adaptive mechanism(s). Given that acetyl groups are the natural substrate of histone acetylation/deacetylation enzymes, the addition/removal of such groups in response to increased or decreased availability may represent a direct way to

introduce nutrient-related epigenetic changes (Figure 4). In this scenario, establishing new acetylation patterns dependent on nutrient availability would serve the double purpose of operating changes in the chromatin landscape that allow the immediate adaptation and survival of the developing embryo while also introducing an epigenetic mark that can be passed down to the daughter cells. Despite histone acetylation being reversible, the epigenetic signature might become fixated in the genome through additional mechanisms, especially when cell plasticity is progressively lost. A similar mode of action can be hypothesized for other histone covalent modifications, such as lactylation.

This metabolic flexibility allows embryos to adjust their energy production and use according to the available resources, enabling them to maintain cellular functions and development under stress. However, while this adaptability is essential for immediate survival and proper development, it may have long-term consequences, potentially influencing the health and viability of the embryo as it progresses through later stages of development and into adulthood (Sharpley et al., 2021). The ability to shift metabolic pathways and adjust cellular functions during early development can leave lasting imprints on cellular behavior, potentially affecting key developmental outcomes, including gene expression and cell fate decisions. The change can be passed to daughter cells, and the altered phenotype can be transmitted transgenerationally through epigenetic inheritance (Perez & Lehner, 2019).

4. Conclusions

This review article summarized the main patterns of histone acetylation during the early stages of embryo development, a time of intense epigenetic remodeling. Our survey highlighted that, despite a relatively conserved program of totipotency and pluripotency among mammals, few reports agreed on common dynamics of histone acetylation. At the same time, several discrepancies were found between the different reports, also when the same species was considered. While variations of the experimental setting and methodologies might partially be held accountable for lack of reproducibility, an emerging theme of our analysis was the capacity of the early embryo to fine tune its metabolism to adapt to changing environments. Notably, histone acetylation participates in such regulation by being the acceptor/donor of acetyl groups - that ultimately are a function of nutrients availability - and as an epigenetic modifier.

In this scenario, the lack of a strict consensus is less appalling, as variations in nutrient availability from the maternal diet or the culture conditions are expected to introduce changes at which the developing embryos are ready to react in light of the inherent plasticity.

If this hypothesis is confirmed, it will be essential to ascertain how permanent these modifications can be and if there are safe ranges in which the embryo tolerates exposure to nutrient defect or excess without impacting the long-term epigenetic reprogramming.

Figure Legends

Figure 1. Dynamic changes in the acetylation of histone H3 during early embryo development. A summary of acetylation patterns at different lysine residues on histone H3 (H3K9ac, H3K14ac, and H3K27ac) across various stages of preimplantation development in mouse, bovine, and porcine embryos is provided. The chart illustrates the presence and intensity of acetylation, represented by shades ranging from pink (least acetylated) to red and purple (most acetylated) at each residue from the zygote to the blastocyst stage. The stages analyzed include zygote, 2-cell, 4-cell, 8-cell, morula, and blastocyst. References for each residue are indicated on the right. Created with BioRender.

Figure 2. Dynamic changes in the acetylation of histone H4 during early embryo development. A summary of acetylation patterns at different lysine residues on histone H4 (H4K5ac, H4K8ac, and H4K16ac) across various stages of preimplantation development in mouse, bovine, and porcine embryos is provided. The chart illustrates the presence and intensity of acetylation, represented by shades ranging from pink (least acetylated) to red and purple (most acetylated) at each residue from the zygote to the blastocyst stage. The stages analyzed include zygote, 2-cell, 4-cell, 8-cell, morula, and blastocyst. References for each residue are indicated on the right. Created with BioRender.

Figure 3. Acetylation of H4K8 and H4K12 in bovine blastocyst. Representative images of hatched or hatching blastocysts stained with DAPI (blue, A, C, E) and negative control (B), AcH4K8 (D), and AcH4K12 (F) in red. Bar=50 μm. Embryos were fixed and stained using specific antibodies, as described in (Franciosi et al., 2012; Lodde et al., 2021) and show a lower immunostaining intensity at the ICM.

Figure 4. Proposed model of embryo metabolism affecting histone acetylation. Glucose metabolism generates pyruvate, which enters the mitochondria and is converted into acetyl-CoA via the tricarboxylic acid (TCA) cycle. Acetyl-CoA is transported to the nucleus where it is used as a substrate for histone acetylation mediated by histone acetyltransferases (HATs). Histone acetylation levels are dynamically regulated by the opposing actions of HATs and histone deacetylases (HDACs), influencing chromatin structure and gene expression. Created with BioRender.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: G.M. and F.F.; Methodology: G.M.; Writing – Original Draft: G.M. and F.F.; Writing – Review & Editing: F.F.F., F.M., A.M.L., and V.L.; Visualization: G.M.; Supervision: F.M., A.M.L., and V.L..; Funding Acquisition: F.M., A.M.L., and F.F.

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Figure 1

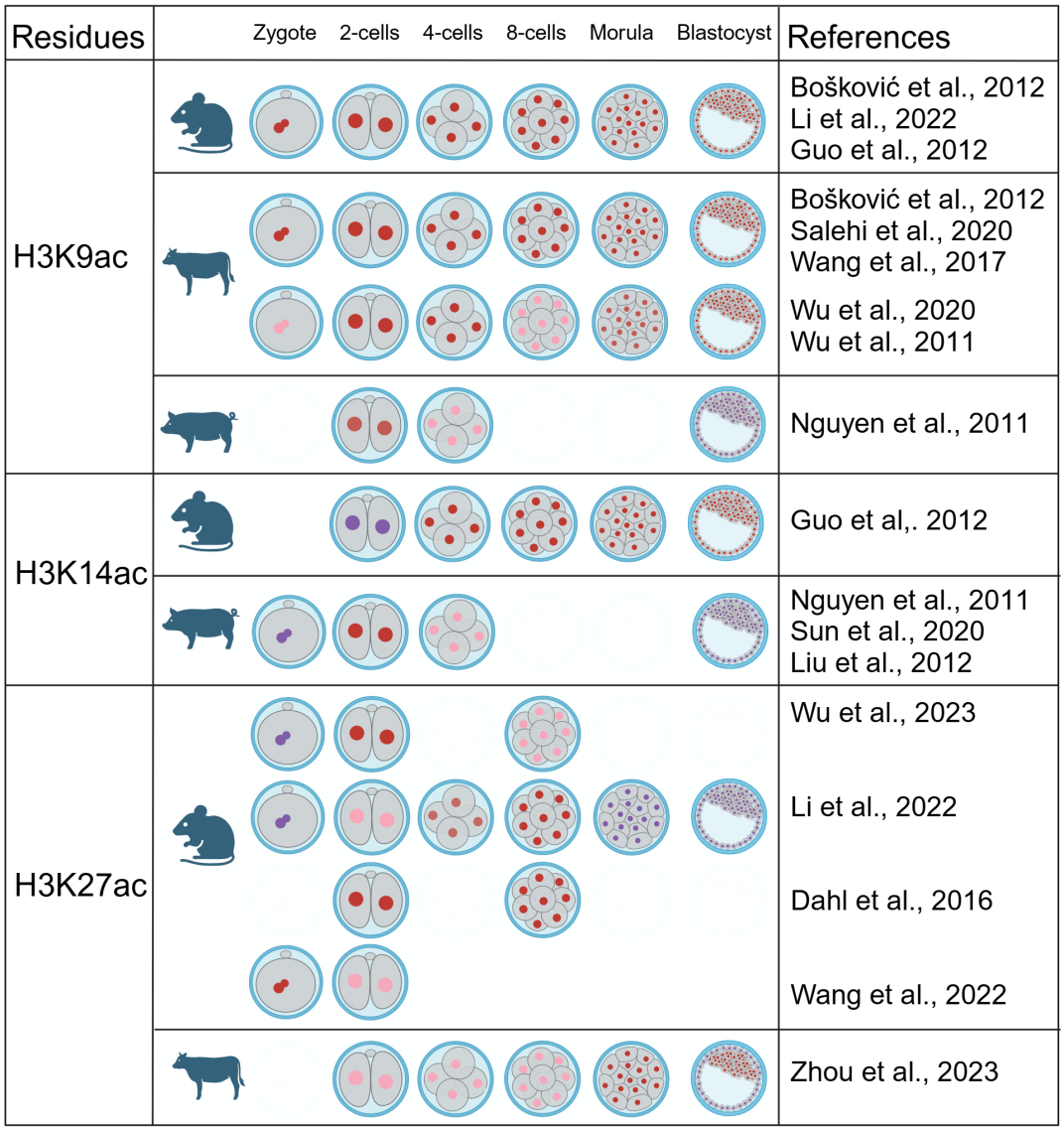
Residues
Zygote
2-cells
4-cells
8-cells
Morula
Blastocyst
References
H3K9ac
Bošković et al., 2012
Li et al., 2022
Guo et al., 2012
Bošković et al., 2012
Salehi et al., 2020
Wang et al., 2017
Wu et al., 2020
Wu et al., 2011
Nguyen et al., 2011
H3K14ac
Guo et al,. 2012
Nguyen et al., 2011
Sun et al., 2020
Liu et al., 2012
H3K27ac
Wu et al., 2023
Li et al., 2022
Dahl et al., 2016
Wang et al., 2022
Zhou et al., 2023

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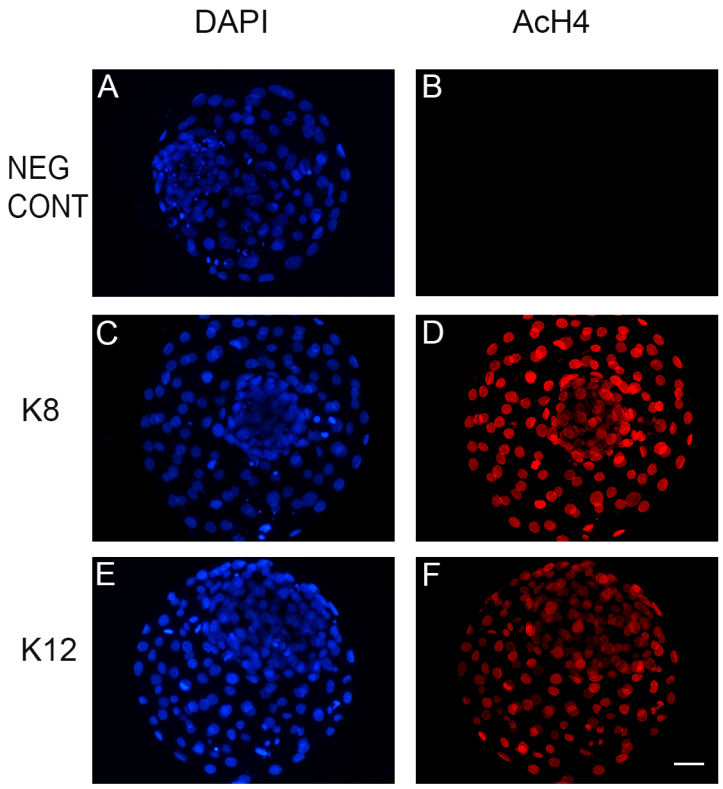
Figure 2

Residues	Zygote	2-cells	4-cells	8-cells	Morula	Blastocyst	References
H4K5ac							Ma et al., 2008
H4K5ac H4K8ac							Wu et al., 2020 Wu et al., 2011
H4K8ac							Cervera et al., 2009
H4K16ac							Cao et al., 2017

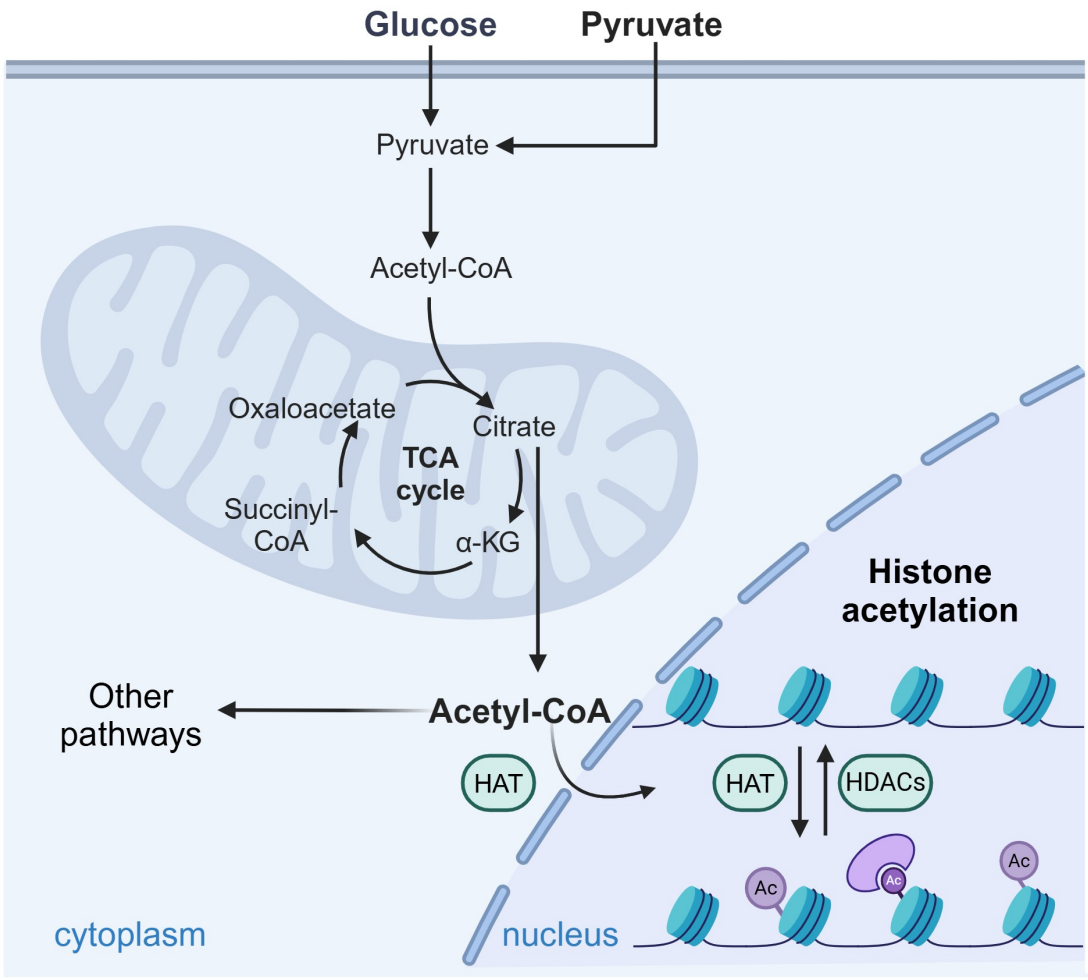
950

Figure 3



955

Figure 4


Glucose
Pyruvate
Pyruvate
Acetyl-CoA
Oxaloacetate
Citrate
TCA cycle
Succinyl-CoA
α-KG
Other pathways
Acetyl-CoA
Histone acetylation
HAT
HAT
HDACs
Ac
Ac
Ac
cytoplasm
nucleus