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Review article

Present and future challenges for the investigation of transgenerational epigenetic inheritance

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ABSTRACT

Epigenetic pathways are essential in different biological processes and in phenotype-environment interactions in response to different stressors and they can induce phenotypic plasticity. They encompass several processes that are mitotically and, in some cases, meiotically heritable, so they can be transferred to subsequent generations via the germline. Transgenerational Epigenetic Inheritance (TEI) describes the phenomenon that phenotypic traits, such as changes in fertility, metabolic function, or behavior, induced by environmental factors (e.g., parental care, pathogens, pollutants, climate change), can be transferred to offspring generations via epigenetic mechanisms. Investigations on TEI contribute to deciphering the role of epigenetic mechanisms in adaptation, adversity, and evolution. However, molecular mechanisms underlying the transmission of epigenetic changes between generations, and the downstream chain of events leading to persistent phenotypic changes, remain unclear. Therefore, inter-, (transmission of information between parental and offspring generation *via* direct exposure) and transgenerational (transmission of information through several generations with disappearance of the triggering factor) consequences of epigenetic modifications remain major issues in the field of modern biology.

In this article, we review and describe the major gaps and issues still encountered in the TEI field: the general challenges faced in epigenetic research; deciphering the key epigenetic mechanisms in inheritance processes; identifying the relevant drivers for TEI and implement a collaborative and multi-disciplinary approach to study TEI. Finally, we provide suggestions on how to overcome these challenges and ultimately be able to identify the specific contribution of epigenetics in transgenerational inheritance and use the correct tools for environmental science investigation and biomarkers identification.

1. Introduction

Environmental cues can deeply impact natural populations through phenotypic plasticity which allows one genotype to express several phenotypes depending on the surrounding environment (Thomas et al., 2016). Medium and long-term regulation of transcriptional processes is controlled by a set of molecular pathways gathered under the term of epigenetics. These processes are key to the development of organisms as well as to maintain physiological functions (Palli, 2020). Epigenetics has been defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence (Dupont et al., 2009)." Nevertheless, what molecular phenomenon exactly is part of epigenetics is still controversial and a matter of extensive discussion in literature (Berger et al., 2009; Howie et al., 2019; Ptashne, 2007). For us, epigenetic processes encompass both epigenetic marks and epigenetic mechanisms. We define as epigenetic marks DNA modifications (Iyer et al., 2016; Feng, 2010), post-translational histone (or protamine) modifications (Kouzarides, 2007), histones variants (Talbert and Henikoff, 2014), histones retention sites in sperm (Ben Maamar et al., 2018), and nucleus architecture

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(Lanctôt et al., 2007). On the other hand, we define epigenetic mechanisms as all processes involved in the establishment of epigenetic marks or responsible for mitotically stable changes in gene expression, such as post-transcriptional regulation by non-coding RNAs (ncRNAs) (Peschansky and Wahlestedt, 2014). Finally, we consider as epigenetic drivers all the epigenetic processes that are involved in the ontogeny of a specific phenotype.

Epigenetic processes are tightly regulated during development and respond to changes in levels of endogenous and exogenous factors. They are sensitive to a large variety of environmental factors such as, diet (Nica, 2017), pathogens (Ottaviani, 2013), and chemicals (Pierozan et al., 2020; Ding, 2015; Aluru, 2017), physical parameters like temperature and salinity (Liew, 2020), as well as psychosocial factors such as parental care (Hur et al., 2017). In addition, some epigenetic modifications are stable through meiosis and can therefore influence future generations. This phenomenon is known as transgenerational epigenetic inheritance (TEI), whereby TEI is defined as the "germline-mediated inheritance of epigenetic information between generations in the absence of direct environmental influences, that leads to phenotypic variation" (Skinner, 2011; Nilsson et al., 2018). In the last two decades, consensus has grown on the idea that biological factors transmitted from parent to offspring include not only genetic but also epigenetic contribution (Adrian-Kalchhauser, 2020; Xavier et al., 2019; Cosseau, 2017). The regulation of the epigenome is more dynamic than processes that are determined by DNA sequences, and, as such, they are expected to be more sensitive to a wider array of environmental cues and spread more quickly within a generation (Rando and Verstrepen, 2007; Danchin, 2011; Richards et al., 2012). Therefore, epigenetic changes allow to increase phenotypic diversity and for faster adaptation to rapid environmental changes (Rey et al., 2016; Klironomos et al., 2013). For instance, epigenetic changes have been associated with adaptation of plants to stress (Mirouze and Paszkowski, 2011), adaptation of invasive species (Ardura et al., 2017), or organisms' acclimation to ocean warming (Ryu et al., 2018). On the other hand, they were also proposed to result in maladaptation and adversities in unexposed generations following a parental stress (Sarkies, 2020). Investigations on TEI are therefore highly relevant to adaptation and evolutionary processes as well as toxicological effects.

During the last two decades, there has been an increasing number of studies and publications reporting transgenerational effects in combination with epigenetic modifications. However, the contribution of TEI to natural evolutionary processes (both adaptative and neutral) is still controversially debated among the scientific community, partly because of the biological and technical difficulty to isolate the primary epigenetic contribution from genetic and environmental factors underlying heredity (commented by (Horsthemke, 2018) and (Husby, 2022) but also because it is unclear how much TEI contributes to the offspring phenotype. In addition, some studies showed that epigenetic modifications are completely erased after a certain number of generations, suggesting that epigenetic changes may only support short-term adaptation rather than evolutionary processes (Cropley, 2016). However, germline epigenetic changes may also have long-lasting evolutionary effects by biasing mutation rates in single nucleotides or regulating the emergence of copy number variations (Guerrero-Bosagna, 2020). Overall, the isolation of inherited effects from intragenerational plasticity is complex and requires methodological rigor (Johannes, 2009; Bell and Hellmann, 2019; Burggren, 2015). The present article aims at describing the major challenges associated with the study of TEI and propose strategies to fill the identified knowledge gaps.

2. Challenges in epigenetic research

In addition to their specific challenges, the study of TEI is facing similar difficulties as encountered in any epigenetic research: lack of investigations about the interplay between the different epigenetic actors, difficulty to link epigenetic changes to an outcome, and limitations related to methodology and data analysis.

2.1. Study of the interplay between epigenetic mechanisms

The collection of all epigenetic marks constitutes the epigenome, with a complex interplay between each layer (Danchin, 2011). Because of species differences in epigenome constitution, there has been a strong bias toward the study of a specific epigenetic process in different species. For instance, in vertebrates and mollusk species, most focus has been on DNA methylation (DNAme) followed by histone modifications. Numerous methods have been identified to study these two carriers of epigenetic marks (see (Fallet et al., 2020) for a list of existing methods). In other species where DNAme is low or absent, the focus has been set on ncRNA species (*C. elegans*) (Wenzel et al., 2011) or histone modifications (*Drosophila melanogaster*) (Kharchenko and v., 2011). Nonetheless, epigenetic processes are interconnected. For instance, in mammals, H3K4me2 and H3K4me3 are associated with unmethylated DNA while H3K36me3 is correlated with DNAme presence (Rose and Klose, 2014; Smallwood and Kelsey, 2012). In germ cells, histone modifications are suspected to direct the DNAme machinery (Turner, 2009). Another example is the role of ncRNAs to recruit binding proteins, influence histone marks (Portela and Esteller, 2010; Tuddenham, 2006), and ultimately DNAme (Portela and Esteller, 2010; Lahmy, 2016; Erdmann and Picard, 2020). Therefore, a first limitation in current epigenetic research is that most often the interactions between the different epigenetic mechanisms are barely addressed. However, the difficulty to clearly identify the epigenetic mechanisms involved in TEI has raised the need for the study of their interplay. In that sense, we propose that study designs should address all or most epigenetic layers. Integrative studies looking at two or more epigenetics layers have been conducted and can be taken as examples (Beck et al., 2021; Brinkman, 2012; Kawaguchi and Hirose, 2012; Kim, 2009; Park, 2018). For instance, in rats, both DNA methylation, ncRNAs and differential histone retention sites were studied in response to a vinclozolin or DTT exposure using MEDIP-seq, ChIP-seq and Illumina sequencing. Results showed a co-localization of the three epigenetic markers to the same chromosomal regions and suggest their integration into an RNA-directed DNA methylation and a DNA methylation-directed histone retention process[54].

2.2. Correlating epigenetic changes to their outcomes

In many studies, epigenetic modifications are not associated to outcomes before reaching conclusions about the impact of epigenetic modifications on the phenotype of an organism (Blanc, 2021; Kamstra et al., 2017). In fact, as often claimed by ecological and evolutionary sciences not all changes in epigenetics are adverse, with some of them being neutral from the standpoint of fitness (Guerrero-Bosagna, 2017), and others described to be adaptive (Kronholm and Collins, 2016; Kronholm et al., 2017). Additionally, some epigenetic changes may not cause further physiological consequences and remain silent (English et al., 2015). Even when epigenetic drivers are identified, it is difficult to establish a link between their modification and a specific outcome. For instance, DNAme at CpG sites and gene expression were initially thought to be negatively correlated (English et al., 2015; Razin and Cedar, 1991). However, accumulating evidence shows that this correlation is highly dependent on the genomic location of the differentially methylated region (i.e., promoter, gene body, intergenic region) (e.g., (Falisse, 2018; Jones, 2012) and sometimes results can be inconsistent with the commonly admitted pattern (Li, 2015). Changes in DNAme within functional intergenic regions are particularly difficult to correlate with gene expression patterns (Blanc, 2021; Kamstra, 2018), which is limiting the identification of direct relationships between epigenetic and physiological changes (Vanderkraats et al., 2013; Jjingo et al., 2012).

Theoretically, the identification of epigenetic drivers would require investigating epigenetic processes as a whole to be able to isolate their respective contribution to the phenomena (Bošković and Rando, 2018).

In this regard, validation studies are critically lacking in the field, although they are necessary to establish causal links between epigenetic dysregulation and a specific (inherited) phenotype (Liberman et al., 2019). For example, paternal stress has been investigated and injection of a specific subset of 9 sperm miRNAs into the oocyte recapitulated the observed inherited behavioral defects, which validate the role of miRNAs as epigenetic drivers for TEI of paternal stress (Rodgers et al., 2015). The ongoing development of locus-specific epigenome editing tools (such as restriction enzymes, zinc finger nuclease or CRISPR-cas9) should favor the identification of causal relationships within the next few years (Horii, 2020; Yim et al., 2020).

2.3. Using appropriate methods

2.3.1. Appropriate model

The research question will define the choice of the studied organism, and model organisms are often preferred for transgenerational epigenetic studies. For instance, the nematode *C. elegans* is one of the models of choice to investigate TEI as, thanks to its fast generation time and its self-fertilization reproduction, a high number of generations can be investigated, and isogenic lineages can be obtained (Minkina and Hunter, 2018; Woodhouse and Ashe, 2020). In addition, the study of epigenetic biomarkers in mice is commonly used to get insight into the development of diseases such as cancer (Walrath et al., 2010). Identification of epigenetic biomarkers on sentinel species like mollusks was suggested for marine pollution and chemical risk assessment (Jeremias et al., 2020); (Rondon, 2017). For a deeper understanding of epigenetic and TEI mechanisms in nature, studies on a wider array of species, not only in model species, should be performed. Of course, the studied organism must be chosen wisely in regard to the research question (as it will directly impact the available epigenetic mechanisms), the number of generations needed to detect a transgenerational effect (see part 2.2.), and the cost of the study. In order to avoid blind experiments and the use of hard to handle non-model species that might be costly and difficult to maintain in laboratory, we suggest at first to perform comparative studies among already available data and to identify the common and specific mechanisms that could later be more specifically addressed in other species of interest. Noteworthy are the efforts towards the development of in vitro models for epigenetic studies. For instance, cells culture has been used to investigate epigenetic involvement in cancer, and if 2D cultured cells show some dissimilarities with in vivo cells, 3D culture systems may help to resolve that issue and have been shown to have biological profiles closer to the primary tumor cells (Rogers, 2018). Even more promising for TEI are the use of pluripotent stem cells and reprogrammed cells models allowing to mimic the in vivo developmental pathway to study germ cell differentiation and early processes after fertilization in vitro (Gonen, et al., 2021; Hayashi and Surani, 2009). Such technics are promising notably for the study of epigenetic reprogramming and reprogramming resistant epigenetic alterations in homogenic cell populations.

2.3.2. Appropriate sampling

In TEI studies, sampling must be cautiously designed and performed to be able to investigate the history of epigenetic marks that may underlie an inherited phenotype. This is particularly important in the case of investigations involving developmental stages. Especially during reprogramming, organisms and single cells undergo fast and massive epigenetic changes e.g., changes in chromatin structure and DNA accessibility (Schulz and Harrison, 2019). Accordingly, sampling procedures in early developmental stages must be carried out with particular attention and capture “epigenetic frames”, which we define here as time points when epigenetic processes act on specific marks and consequently define modifications for an extensive epigenetic reorganization. We propose that a dynamic analysis of broad epigenetic features, and identification and characterization of epigenetic frames is fundamental for understanding the process of establishment of specific phenotypic traits, especially across generations. According to that, sampling procedures in early developmental stages must be selected and performed with particular attention. For instance, in one of the most used animals for environmental studies, i.e., the zebrafish, the sampling procedure of embryos is usually based on the hours post fertilization (hpf) (Yesudhason, 2020), which leads to missing the first developmental stages which occur quickly and progressively in the first hour. Such approach should not be used in TEI design especially when spawning and fertilization are out of synchronization. Importantly, if the study design involves natural mating, eggs may be fertilized at different times and the sampling will correspond to mixed cell stages. Thus, the application of the standard design can result in a mixture of epigenetic frames in which epigenetic features cannot be properly discriminated and studied. Additionally, even the collection of samples at the same cell stage could lead to low sampling resolution. For instance, in some species such as mammalians, the same cell stage can have a long duration but still include intensive chromatin reorganization (Chen, 2019). To reduce this problem, two strategies may be considered according to the species and lab equipment: 1) manual or machine-learning sorting based on morphology and 2) the use of in vitro fertilization to synchronize early embryonic stages (Breitwieser et al., 2018; Neukum, 2019).

2.3.3. Tissue heterogeneity

In addition to getting the adequate model and performing sampling in statistically meaningful numbers, tissue heterogeneity can also be an issue (Bakulski et al., 2016). Indeed, the epigenome is highly dynamic even within a tissue, and disentangling epigenetic effects between heterogenous cell populations inside a tissue can be challenging. For instance, blood is a highly heterogenous tissue and comparing differential DNA methylation patterns between blood samples of different individuals could mislead to wrong conclusions about individual epigenetic differences which would indeed be due to differences in samples cellular composition (Heijmans and Mill, 2012). Single-cell sorting, e.g., FACS, laser capture microdissection (LCM) and celleNONE, can be used for sorting specific cell populations and understanding trajectories leading to a specific phenotype even later in development. Such single cell technologies can even be used to distinguish among cells subtypes (Liau, 2023) but have some limitations such as they may induce stress on cells and thus alter epigenetic profiles (Hu et al., 2016). In addition, FACS requires a high number of cells (more than 10 000), while LCM can be time consuming because of the necessity to identify cells of interest through visual microscopic inspection, besides introducing technical artifacts as UV damage to DNA or RNA from the laser cutting energy[91]. Next to single-cell isolation techniques, development of single cells analysis can allow to decipher between cells type without stress induction. Single cells spatial transcriptomics and single cell proteomics together with adapted computational methods can be used to study spatially resolved gene expression and post-translational modifications of diverse cell types in complex tissues (Hu et al., 2016; Chidester et al., 2023). Besides, as for any highly dynamic system that cannot easily be studied in situ, it is crucial to ensure sufficient sample numbers so that even small changes can be observed, and statistically relevant results can be produced. In addition, cross-tissues studies must be encouraged to allow the creation of integrative maps of epigenetic mechanisms in whole organisms.

2.3.4. Appropriate technology

Methodologies to investigate epigenetics are in constant development. They have evolved from global to local genomic interrogation with single nucleotide resolution, using both targeted and genome-wide approaches. Furthermore, they have been adapted to also capture stable as well as transient marks, the latter with the help of biochemical modifications such as (photo)crosslinking for capturing transient protein–protein interactions (Zhang, 2022).

DNA methylation, specifically 5mC, is an epigenetic mark extensively studied in the environmental field (Kamstra et al., 2015; Keil and

Lein, 2016). Although global measurements of DNA methylation were fundamental to uncover large changes in DNA methylation, for example in relation to cancer (Soares, 1999), they are insufficient to capture regional changes in methylation and explain the role in contributing to the information carrier phenotype (Lisanti, 2013). Local assessment of epigenetic status, instead, involves measuring marks in specific regions of the genome and allows for the identification of modifications at high resolution. There are numerous technologies that will allow the measurement of specific layers of epigenetic organization. To study specific DNA methylation, for example, DNA methylation bisulfite sequencing-based technologies are often used and include e.g., Whole-Genome Bisulfite Sequencing (WGBS) and Reduced-representation bisulfite sequencing (RRBS-Seq). Bisulfite sequencing involves the in vitro chemical conversion of unmethylated cytosines to Uracil, which after sequencing allows to differentiate methylated cytosines, maintained as cytosines, from unmethylated cytosines, sequenced as thymidines (Clark et al., 1994). Methylated DNA Immunoprecipitation (MeDIP), in turn, involves the enrichment of the methylated fraction of a DNA sample via capture with using an anti 5-methylcytosine antibody (Weber, 2007). The method was initially developed for microarrays but then it was implemented with sequencing (Ruike et al., 2010). MeDIP is also commonly used and provide larger genome coverage and depth (Jacinto, 2008). Newer methods such as Anchor-based bisulfite sequencing provide fast results at the base resolution and allows to work with a lower number of reads (Chapin et al., 2022). Although these analyses are extremely valuable in the case of biomarker identification, they are limited to a single mark and may be unsatisfactory to explain how phenotypic information is inherited across generations. As we discussed, it is likely that the establishment of a phenotype involves interactions among different epigenetic actors, including ncRNAs and histone marks. Luckily, methods for the parallel investigation of different epigenetic mechanisms already exist. For instance, sequential ChIP-bisulfite sequencing can be used to get insights on the DNA methylation and histone modification crosstalk (Brinkman, 2012). Yet, starting material needed and the high costs for multiple technologies limit study design for multiple marks. In this context, single cell technologies have gained much attention through their ability to measure multiple levels of biological information, i.e., transcriptional (single-cell RNA sequencing) and chromatin features (CUT&Tag), on a limited amount of material, and may be applied to reduce the sampling procedures (Belhocine et al., 2021; Kaya-Okur, 2019). Less costly alternatives include CyTOF applications (Tracey et al., 2021), which can measure several single targets simultaneously and can be combined across all levels of the biological hierarchy. CyTOF uses elemental metal isotopes (Kay et al., 2016) linked to monoclonal antibodies to measure several parameters simultaneously in single cells. It uses the concept of flow cytometry and mass spectrometry to measure the number of antibodies bound to a specific target which represents the expression level of the target antigen (Ornatsky, 2010). But it is likely that their high cost and challenging experimental processing workflow may still be limiting.

Even more promising are also third-generation sequencing methods based on voltage changes i.e., Oxford Nanopore Technology (ONT) (Kono and Arakawa, 2019; Bibikova, 2006). Indeed, this new technology can analyze DNA and RNA modifications on multiple regions of the same sample without pre-treatment, i.e., bisulfite chemical conversion or use of antibodies, since the data are generated on long sequences of native nucleotides passing through a nanopore. Based on this, ONT can potentially analyze several different epigenetic features simultaneously, i.e., DNA methylation (5mC, 5hmC, 6 mA), RNA methylation (6 mA) and ncRNAs (Parker, 2020). The ONT can be used to sequence strands ranging in length from 70 to 26,000 nucleotides with good confidence. Several reports even show longer read lengths but may suffer from quality issues. Therefore, long non-coding RNAs, with a length between 1 000 and 10 000 nucleotides, are easily characterized using this approach. Small non-coding RNAs whose length ranges between 18 and 200 nucleotides, are more challenging to be measured using the standard ONT approach. Efforts towards enabling the characterization of non-poly(A) short RNA species is ongoing as seen by the establishment of NERD-seq (Saville, 2021) but are still early in development. However, ONT has been used for the analysis of transfer RNAs (tRNAs) (Thomas, 2021) that are known to play a role in epigenetic regulation of chromatin conformation in mammals (Ebersole, 2011) and Arabidopsis (Hummel, 2020), and that are susceptible to play a role in TEI in rats (Schuster et al., 2016) and *C. elegans* (Fallet et al., 2023, *work in progress*). All the analysis can be performed using the same device and raw data can also be used to address genetic changes i.e., mutations, which should be analyzed in TEI studies to clarify whether a phenotypic change is due to genetic or epigenetic cues. Interestingly, this approach can be used for multiple species such as vertebrates and invertebrates (Dimond et al., 2021), including the ones lacking in 5mC as other marks can be detected (Roach, 2020). Moreover, since it is a long-read method, complex genomes are easier to be analyzed than with short read methods. Thus, such method can be developed as a universal approach. However, this technology needs to be improved since the error rate is higher than other sequencing methods (Rang et al., 2018). Lastly, unlike the short read sequencing methods e.g., Illumina, the analysis of non-canonical nucleotides is performed on native nucleotides, therefore ONT cannot be applied to limited starting material or single cell level because it does not include any amplification step. Finally, another interesting approach is photo-cross-linking, which can be used to investigate protein–protein, protein–nucleic acid, and protein–small molecule interactions involved in epigenetic processes regulation. For instance, photo-cross-linking can identify specific histone modifications and their associated interacting writer and eraser proteins (Zhang, 2022). This technique seems promising for future studies on epigenetic interactome in specific chromatin context (Zhang, 2022).

To conclude, in the absence of a whole epigenome characterization technology, despite the great potential of newly developing technologies, the selection of the most suitable methods to measure the various levels of epigenomic change regarding the biological question and the species of study remains crucial.

2.3.5. Data analysis

As with all large datasets in the biological sciences, epigenetics also comes with a varying number of analysis pipelines and techniques. Just as in other OMICs, the use of different preprocessing strategies and/or statistical tests can lead to different interpretations of the studied system. For example, numerous sequencing processing methods are available with varying assumptions and outcomes (Breton et al., 2021; Corchete, 2020). In addition, the number of novel single-cell approaches is growing exponentially. Challenges in processing, imputation (or rather feature smoothing), batch correction, and interpretation of the data play a key role in these developments (Gao, 2021). In parallel, sequencing depth can also be a challenge. Approaches such as Reduced representation bisulfite sequencing (RRBS), for example, are often used within ecotoxicology due to the lack of funding to perform whole genome sequencing approaches on the required number of samples for statistical analysis. Such reduced representation approaches have the benefit of allowing for increased coverage of the reduced regions of the genome investigated. Additionally, the low cost of these technologies allows for an increased number of biological replicates to be included, which favors statistical power. While it is economically more viable the resulting data can be significantly different when multiple runs are required or have been performed on different days. Further comparison to other datasets is also limited as not all regions have been measured equally. According to that, we suggest selecting technologies allowing the most complete sample characterization making these data useful for later comparison.

A big challenge in all high throughput data generation techniques is the concept of batch effects which can occur because of technical differences between different physical machines, differences between the human handlers, as well as differences resulting from the time of the

experiment. Batch effects can be challenging to remove, particularly when they do not correspond to the available metadata. Several techniques have proposed to deal with batch effects including ComBat(-seq), linear mixed effect model, or Passing-Bablok regression approaches (Luo, 2010; Tran, 2020; Müller, 2016). While batch effects often are uncharacterized, they can play a significant role in downstream analyses if not considered.

Once the underlying data have been processed and quality checked, downstream analyses help interpreting and understanding the biological system. Here it is important to understand the assumptions underlying the statistical test, the predictive model, or any of the other types of analyses that are performed on the data. Selection is often driven by how well we understand the system, how much data is available, and how reproducible a particular dataset is. Importantly, while power analyses can help guide which analysis can be performed with which data or how many samples would be required to achieve sufficient power, they are often designed in a one-size fits all situation if the underlying parameters of variance or reproducibility are not known. The use of unpowered experiments can lead to misleading conclusions, particularly in relation to the confirmation of epigenetic changes (Guerrero-Bosagna, 2016). It is therefore advisable to include an experienced bioinformatician early into the experimental design to ensure that an appropriate number of biological replicates is included, the required biological questions can be answered, and the desired experimental outcomes can be achieved.

Data interpretation, particularly when considering DNA based sources, is challenging. While we understand more and more about the different genomic regions across many species, we still lack the functional explanation for many of these. For example, while we understand that there are interactions between intergenic regions, these are often not taken into consideration when analyzing and interpreting epigenetic information. Instead, focus is given to coding regions where some functional information is available. Approaches such as the reconstruction of the 3D structure of the DNA (Varoquaux et al., 2014) can help in this regard but its use and development of techniques is still largely lacking.

3. Epigenetic mechanisms in inheritance processes

3.1. Mechanisms underlying reprogramming and imprinting

Early-life development entails multiple waves of epigenome-wide reprogramming, *i.e.*, erasure of most germ-cell specific epigenetic patterns and replacement by embryo-specific marks (Cantone and Fisher, 2013). In mammals, the epigenetic landscape undergoes two known waves of reprogramming, (1) at zygotic genome activation and (2) at germ cell differentiation (Morgan et al., 2005). Thus, histone modifications, histone variants, and DNA methylation are reset along the genomic sequence, except for regions subjected to parental imprinting (Cantone and Fisher, 2013; Zilberman and Henikoff, 2005) as well as some virus-induced RNA silencing and transposable element silencing (Zilberman and Henikoff, 2005). In addition, for yet unknown reasons, some regions have been shown to escape reprogramming and can thus persist over generations in an imprinted-like fashion (Jablonka and Raz, 2009; Richards, 2006; Tang, 2015). It is of importance to notice, however, that research on reprogramming and imprinting events has limited extrapolative power as these are highly variable between clades and even between species (for examples, see (Kawashima and Berger, 2014; Kubo, 2013; Wang and Bhandari, 2019; Balasubramanian et al., 2019; Stancheva et al., 2002; Hughes, 2014).

Currently, there is, to our knowledge, no consensus on why and how some marks are transmitted while others are erased (Hughes, 2014). In vertebrates, the research focus has been set on DNA methylation (DNAme), with some studies suggesting that DNAme marks could be directly transmitted (Anway, 2005; Guerrero-Bosagna and Skinner, 2009; Crews, 2012; Kremsky and Corces, 2020). This is supported by a recent study showing that binding of transcription factors to specific methylated DNA sequences could protect them from demethylation during embryonic development (Kremsky and Corces, 2020).

Nonetheless, DNA methylation reprogramming observed in mammals is not universal which strengthens the fact that wisely choosing the model of study and knowing its biology is critical. Examples from fish species shows a wider variety of reprograming patterns. In the zebrafish embryos, for example, the maternal and paternal methylomes do not endure global demethylation after fertilization. Only the methylation pattern of the maternal genome is reset to match the paternal methylome which is left unchanged (Jiang, 2013). The inherited sperm methylome then participate in the embryogenesis (Skvortsova, 2019). No additional genome-wide methylation erasure happens until sexual maturation (Ortega-Recalde et al., 2019). In contrast, the medaka embryos undergo two global demethylation and remethylating waves similar to what is observed in mammals (Wang and Bhandari, 2019).

In species undergoing DNA methylation resetting, an alternative hypothesis has been proposed for the inheritance of epigenetic marks: that DNA methylation is erased but heritable epigenetic marks generate a signal allowing their re-establishment in the next generation (Baulcombe and Dean, 2014; Calarco, 2012). In that sense, circulating RNAs have been suggested to act as an information carrier for (re-)establishing epigenetic patterns in plants and invertebrates (Baulcombe and Dean, 2014); (Rechavi, 2014). For example, in plants, siRNAs (small interfering RNAs) generated by transcription of active transposons are transported into the germ cells of pollen and further into the fertilized egg to guide epigenetic mark establishment (Slotkin, 2009); (Dunoyer et al., 2013). In drosophila, maternally inherited cytoplasmic piRNAs (piwi-interacting RNAs) can mediate paramutations, i.e., heritable epigenetic modifications induced by one allele of a locus on the other allele without modifying the DNA sequence (de Vanssay, 2012). The existence of this phenomenon in mammals is corroborated by a few studies showing that some ncRNA species can be transferred between generations (Sharma, 2017; Chen, 2015), although their mechanistic involvement in guiding epigenetic marks remains understudied. A study showed that exposure of gestating female rats to vinclozolin leads to changes in DNA methylation and ncRNA expression, as well as modified histone retention sites in sperm of three following generations, with generation-specific patterns (Ben Maamar, M., 2018). A subsequent study carefully investigated the interactions between these three epigenetic processes and showed that half of the differentially methylated regions were conserved from F1 to F2 and F3 (Beck et al., 2021). The authors also observed a correlation between F1 sperm ncRNA profile and F1, F2 and F3 identified differentially methylated regions (DMRs) after both DDT and vinclozolin exposure. Altogether, these findings suggest that ncRNAs can direct DNAme, and further investigations on the involvement of ncRNAs in inheritance are likely to substantially increase our understanding of the mechanisms underlying the conservation and erasure of epigenetic marks during fertilization and embryonic development.

Besides ncRNAs, the role of histone marks in mediating epigenetic inheritance has been highlighted in invertebrates (Xia et al., 2016; Kishimoto et al., 2017) and suggested by a study in mammals (Ben Maamar et al., 2018). Histone methylation of sperm cells may act as an information carrier involved in epigenetic inheritance. In fact, dysregulated histone methylation patterns (H3K4me) in human sperm were linked to immature spermatozoa (Štiavnická, 2020), as well as to altered RNA profiles and histone methylation marks over several generations of offspring in mice (Siklenka, 2015). Altogether, a handful of studies have suggested that changes in the combination of DNAme, histones marks and ncRNAs are driving transgenerational transmission of environmental impacts on the phenotype.

3.2. Investigating an appropriate number of generations

There are several studies showing biological effects in the offspring following parental stress, where the results are mistakenly interpreted as

TEI. In fact, the offspring generation in which a transgenerational effect can be first observed is at least the second (F2) or the third (F3), depending on the environmental factor of interest, the species under investigation, and the experimental design of the study (described in detail in (Valles, 2020). There is no one-fits-all study design in relation to TEI, as the appropriate number of generations to study highly depends on the stressor of interest (e.g., stability, bio accumulative properties), on the window of exposure (early-life stages, reproducing adult), and on the species investigated (e.g., external, or internal fertilization). A true transgenerational effect is observed only when transmitted until the first generation that is not subjected to any direct environmental exposure (Fallet et al., 2020; Skinner, 2008). For instance, in the case of species with external fertilization (e.g., fish), the appropriate number of generations to investigate to identify a transgenerational effect is 3 or 4 depending on the properties of the stressor and window of exposure. In fact, when the F0 generation is exposed to an environmental stressor as reproducing adults, germ cells/gametes leading to F1 generation are also directly exposed (direct effect). The F2 generation will be subjected to indirect exposure resulting from possible epigenetic modifications in F1 germ cells due to F1 early-life exposure (multigenerational effect). The F3 generation is thus necessary to ascertain any transgenerational effect.

If exposed shortly as early-life stages, the F1 generation will only be subjected to an indirect exposure, and a transgenerational effect can be observed in the F2 generation. In species with internal fertilization, the F3 generation is most often necessary. In fact, in mammals, the environmental stressor experienced by the female can be directly transmitted in utero to the fetus (F1) and to the F2 generation through a modification of the F1 fetus germ cells. It is therefore crucial that any conclusion about TEI is based on studies with the appropriate number of generations based on biological knowledge. So far, several authors used the term transgenerational effects without careful consideration of these aspects (Green et al., 2016; Yue, 2013; Salinas and Munch, 2012). In addition, only a limited number of studies followed and identified the inheritance of specific epigenetic marks beyond the necessary number of generations thus limiting our knowledge on the transgenerational stability or loss of epigenetics marks. It is however noteworthy that epigenetic inheritance was shown to be able to last for 50 generations in Drosophila (de Vanssay, 2012).

3.3. Isolating the epigenetic contribution

The epigenetic interplay works in concert with genetic mechanisms; however, the distinction between their respective contribution to environmentally driven inherited phenotypes remain unclear (Horsthemke, 2018; Burgio et al., 2018). A recent method has been reported that combines the interrogation of the genetic and methylomic fraction of reduced individual genomes (Rezaei, 2022). However, studies investigating the transgenerational dynamics between genomic and epigenetic changes are still needed. Most studies assume that transgenerational effects induced by non-genotoxic or non-mutagenic factors are likely the consequence of epigenetic rather than genetic inheritance (Burgio et al., 2018). However, these studies have their limitations as they are not considering that epigenetics and genetics interact and affect one another (Guerrero-Bosagna, 2020; Shen and Laird, 2013). For example, mutations in genes that code for enzymes like DNA methyltransferases, histone deacetylases or methylases can lead to epigenetic changes related to various cancers (Han et al., 2019; Berdasco and Esteller, 2013) or neurological disorders (Berdasco and Esteller, 2013). In addition, ncRNAs are also transcribed from DNA and can be affected by mutations (de Almeida et al., 2016). Moreover, DNAme appears to be, at least partly, genetically encoded *in cis* by small DNA-binding motifs and small methylation-determining DNA regions located inside promotor elements (Lienert, 2011). In consequence, it may be tricky to detangle between genetic and epigenetic carriers of information. Epigenetic changes can also act as enhancers of genome instability. In fact, all common DNA bases can be chemically modified, e.g., methylated, and this can affect interchanges and mutation rates (reviewed in (Guerrero-Bosagna, 2020). For instance, methylated cytosine shows a higher mutation rate than unmethylated one, which has led to a depletion in CpGs during evolution (Xia et al., 2012). There is stochastic epigenetic variation that naturally acts as a driving force for evolutionary adaptation and disease development (Cherry, 2018). In this regard, it seems difficult to exactly determine the contribution of any measured base methylation or other chemical modification, to the development of a specific phenotype. Hence, a recommended prerequisite to investigate the adaptive impact of epigenetic modifications is the measurement of both genetic and epigenetic mutations. This could be initiated by focusing on genetic mutations shown to be important for health followed by the assessment of the implication of epigenetic modifications at the same specific sites. New technologies such as Third generation sequencing, e.g., Nanopore sequencing, offer the possibility to combine the investigation of epigenetic and genetic changes. The development of such tools is essential to decipher the role of epigenetics in the response of organisms to a dynamic and changing environment (Burggren and Mueller, 2015; Stajic and Jansen, 2021).

4. Identification of relevant drivers for TEI

4.1. Background variability and heterogeneity

Epigenetic processes regulate gene expression and thereby influence cell transcriptome and activity (see (Lawrence et al., 2016; Felsenfeld, 2014; Robertson, 2005) for reviews on epigenetic mechanisms). As such, epigenetic changes observed in organisms in response to different stressors could represent early molecular events leading to a specific physiological outcome (Norouzitallab et al., 2019; Willett, 2018). As mentioned before, investigating TEI is complex. Studying parental effects on several generations of offspring carried by epigenetic processes theoretically means isolating a few molecular epigenetic key-players from a wide array of molecular and biological changes, which is experimentally and analytically challenging. A first limitation to consider is the high inter-individual variability of epigenetic landscapes. Besides, epigenetic changes are dynamic, cell- and time-specific, which altogether increase the difficulty in relating specific epimutations to physiological alterations. Further, it was shown that developmental stages are particularly sensitive to epigenetic modifications (Faulk and Dolinoy, 2011) because of high epigenetic plasticity and sensitivity to external factors (Burggren and Mueller, 2015; Fawcett and Frankenhuis, 2015). Therefore, exposure periods encompassing early life stages may be more prone to lead to long-lasting effects and support inherited patterns. Another degree of complexity is added by the fact that all these kinds of changes and interactions can be sex specific. This also concerns gametes since epigenetic carriers transgenerationally transmitted through the germline may be different between oocytes and sperm cells (Almeida, 2019; Chu, 2018; McCabe et al., 2017).

4.2. Identifying the epigenetic drivers of TEI

As mentioned above, some epigenetic changes associated with a particular environmental stress remain silent and may not cause further physiological consequences. In addition, previous research has shown that epigenetic changes can be deterministic (due to factors that target specific DNA regions), stochastic (driven by random events targeting any DNA region), or collateral (Russo et al., 2021; Guerrero-Bosagna, 2017). The latter represents several endogenous intermediates that are shared between metabolic and epigenetic processes (e.g., NAD, FAD, SAM, acetyl-CoA…). Thus, dysregulation of metabolic processes can modulate the epigenome (Gut and Verdin, 2013; Donohoe and Bultman, 2012). Identifying the persistence of epigenetic changes in the germ line and inheritance across generations may reflect transgenerational effects. Nonetheless, an additional aspect to consider for the identification of

epigenetic drivers of TEI is the reversibility/transfer of epigenetic marks. Some studies have identified "epigenetic signatures" for health prognosis (e.g., cancer development (Mancarella and Plass, 2021) or environmental exposures (e.g., cigarette smoking (Joehanes, 2016). However, as described in section 2, only specific epigenetic patterns can escape the different periods of epigenomic reprogramming during development and be transferred to subsequent generations. An important notion is that epigenetic changes that initiate transgenerational phenotypes in exposed organisms are not necessarily the same epigenetic changes that carry TEI across generations (Alonso-Magdalena et al., 2016). This is a direct consequence of the interplay between epigenetic processes, and the possible transfer of epigenetic information from one actor to another. In such a case, both sets of epigenetic modifications can be considered as epigenetic drivers of TEI. For example, behavioral defects were reported in F0, F1, F2, and F3 generations following early-life stress in mice (Gapp, 2014). The authors showed that dysregulation of sperm RNAs in F0 and F1 contributed to the transmitted effects in F1 and F2 generations. Nonetheless, expression of sperm RNAs in F2 was not affected, and thus cannot directly explain the behavioral defects observed in F3. While this finding encourages the study of the interplay between epigenetic processes, it also calls into question the common approach of looking for persistent changes as the ones most likely to explain long-term effects in TEI research. Besides, other research showed that health outcomes could appear from the F2 or F3 generation following F0 exposure or be generation specific (Bell and Hellmann, 2019); (Beck et al., 2021; Alfonso, 2019). This suggests that the inherited epigenetic marks and/or their interactions with other molecular players (genome, other epigenetic mechanisms, metabolic pathways, and microbiota) over generations will lead to the observed effects that were initiated by silent epigenetic modifications. This supports the need for further studying TEI dynamics (Burggren, 2016), molecular interactions, and how the combination of these different components are contributing to changes over generations or not. Once epigenetic drivers are proposed, the inclusion of validation studies (discussed in section 1) must be prioritized to confirm causal links.

5. Toward a collaborative and multi-disciplinary approach to study TEI

Lately, links between epigenetic changes, adaptation, and/or adverse health outcomes following environmental cues have been reported. These results highlight the importance of epigenetics in environment-phenotype interactions. Currently, one of the most striking questions in biology concerns the duration of epigenetic-mediated environmental effects. Indeed, studies investigating TEI have suggested that epigenetic processes could represent the mechanistic link between exposed individuals and descendants showing transgenerationally modified phenotypes. However, the exact molecular mechanisms involved, and their reversibility, remain unclear. This limited understanding is a direct consequence of the complexity of the various underlying biological processes, unsuitable experimental designs, and/or the limited comparability between studies. In addition, the question of the environmentally relevance of the observed results remains often poorly investigated. Thus, more on-site studies should follow the lab experiments (like in (Fallet, 2022)). This is true especially, knowing that most of the time, in nature, one phenotype can be triggered by not only one external stressor but by an array of them (Wood et al., 2010; Pack, 2014). Fallet et al. proposed a standard experimental procedure for intergenerational effect study in mollusks (Fallet et al., 2020); however, no consensual experimental design favoring inter-laboratory and inter-study comparability has been adopted at the level of the community, which significantly weakens overarching biological conclusions. In this review, we have highlighted major limitations concerning the field of TEI and have made suggestions for future improvements that can be summarized as follows: **first**, understanding why some epigenetic marks escape reprogramming, and the exact mechanisms underlying epigenetic inheritance, are questions that require further investigations. We notably highlight the need for studies focusing on the transfer of information from soma to germ cells, and their maintenance from germ cells to embryo. **Second**, genetics and the diverse array of epigenetic processes need to be studied concomitantly to decipher the exact contribution of epigenetics to the overall inheritance system. Indeed, the complex interplay between epigenetic factors is what leads cells -and by extension organisms- to perform the range of phenotypic options referred to in the literature as "epigenetic plasticity". However, because of the complexity of these mechanisms, one of the first steps is to isolate the epigenetic drivers of TEI. Thus, as a **third** point, future research efforts should aim at the description of the epigenome under regular fluctuating conditions (e.g., considering differences across developmental stages, cell specificity, and individual variability within and between generations). This, with the objective of as a **fourth** point, the systematic use of validation studies to isolate and confirm functionally relevant epimutations in response to stressors, among the background of stochastic epigenetic alterations. We hope that the next global effort in new technology developments and the common desire to increase inter-laboratories and inter-fields projects will provide answers on whether epigenetic inheritance can be studied as a stand-alone mechanism, or if these modifications contribute to increased genomic instability and further lead to genetic changes, the latter being the actual support for passing on information over generations -or a combination of both. To include the above-mentioned suggestions and as a **fifth** and final point, we emphasize the need for well-thought and harmonized experimental designs to increase inter-study comparability and reliability. In that regard, the development of better computational tools to analyze such a vast amount of information should be privileged. Fig. 1 shows an attempt to describe an experimental design that would be suitable to study the contribution of epigenetics to an inherited phenotype.

Addressing these different challenges requires intensive scientific collaboration between researchers with different expertise. Hence, we suggest comprehensive and multi-disciplinary projects that combine the investigations of phenotypical changes over generations with investigations of molecular mechanisms that are underlying inheritance. The early incorporation of data analysts during the project development is essential because a disregard for such competencies could jeopardize the success of a project due to too little statistical power or an inadequate experimental design. Combining project partners specialized in different epigenetic processes, biochemistry, and physiology would enable to perform integrative studies and finally decipher the whole epigenetic machinery underlying TEI. We also suggest the use of collaborative platforms and networks to harmonize experimental designs between studies with respect to the research question but also to improve the reproducibility and transparency of the results. Finally, we believe that it is crucial to share data among researchers and the elaboration of epigenetic databases to support and facilitate future research that is built on previous knowledge.

6. Conclusion

In conclusion, the complexity underlying TEI is a great challenge for the use of epigenetic endpoints in applied biology, notably in the context of environmental and (eco)toxicological issues (Chung and Herceg, 2020; Alyea et al., 2014). It requires the development of accurate experimental and computational methods to study TEI as a collaborative effort to ultimately delineate the importance of TEI for the health and survival of different species. Nonetheless, identification of epigenetic processes in ecologically relevant studies and model species would represent an important step toward the incorporation of epigenetics in e.g., risk assessment frameworks to increase the sensitivity and the predictability of hazard evaluation (Willett, 2018; Angrish, 2010). In fact, epigenetic modifications can testify from past experiences, and be measurable endpoints as prospective marks for long-term and perhaps transgenerational toxicity or adaptation. Thus, they could serve as

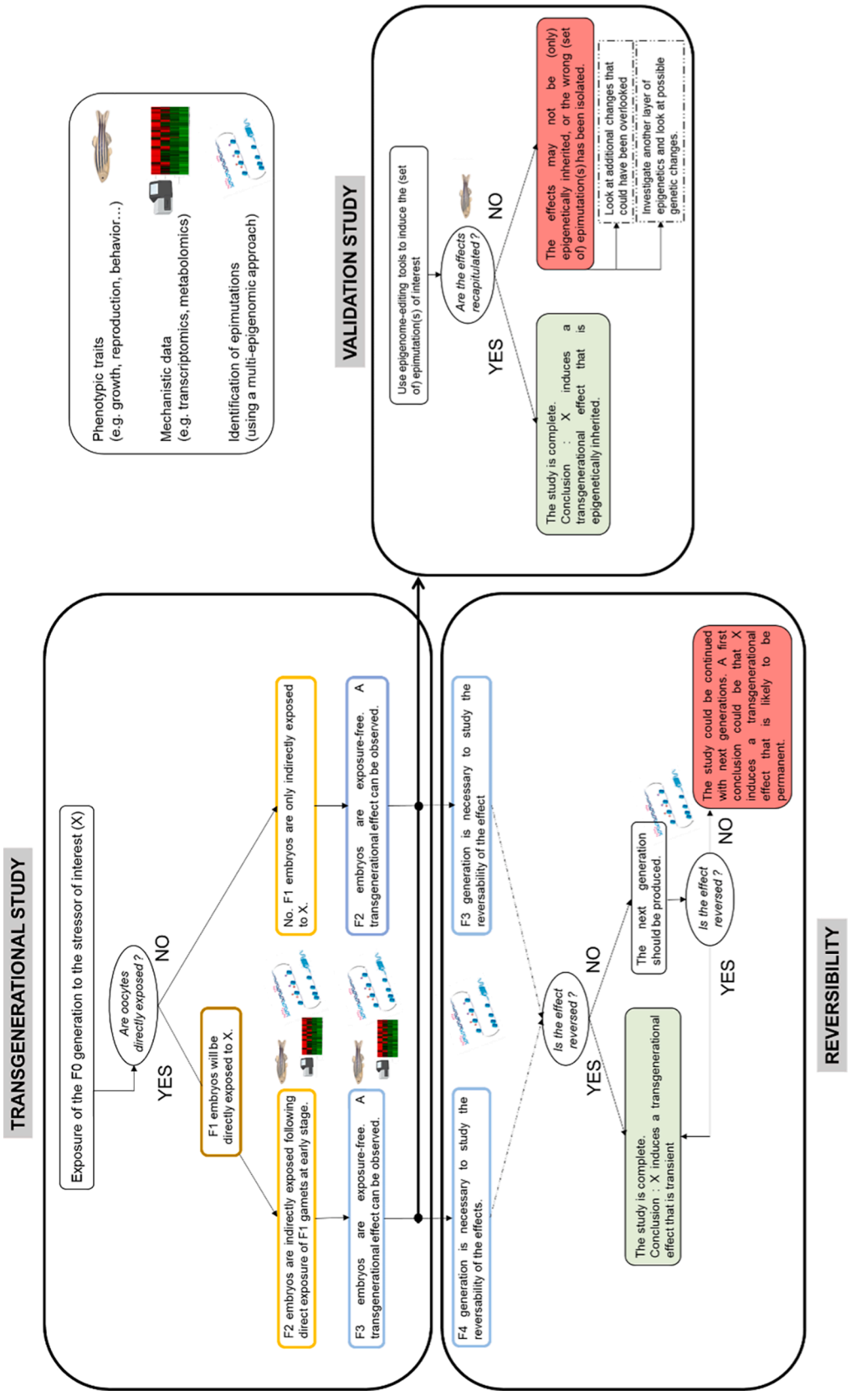

Fig. 1. Suggested experimental design for the study of TE, The F0 generation is exposed to a stressor of interest (X). Depending on the type of stressor, the exposure window, and the model organism, eggs will or will not be directly exposed to X. In the first case, there is the need to include the F3 generation to study transgenerational effects, while in the second case the F2 generation is sufficient. **Transgenerational effects** can be reported as one or several modifications of phenotypic (e.g. reprotoxicity, neurobehavioral toxicity, morphological alterations) and mechanistic traits (e.g. changes in transcriptomic or metabolomic profiles) and should be studied in concert with epigenetic changes. Once a set of transgenerational epigenetic mutations has been highlighted, it is important to include a **validation study** to confirm the key-role of epimutations in the induction of the observed transgenerational phenotype. Finally, examining at least one generation further allows investigations on the **reversibility** of the reported effect across generations. This aspect is particularly important to be clarified with regards to consequences of exposure to X on population fitness in an evolutionary perspective.

biomarkers for environmental impact detection (Manikkam et al., 2012), disease treatment (Jeremias et al., 2020), as well as for species conservation (Rey, 2020).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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