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Review

Chromatin specialization in bivalve molluscs: A leap forward for the evaluation of Okadaic Acid genotoxicity in the marine environment

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ABSTRACT

Marine biotoxins synthesized by Harmful Algal Blooms (HABs) represent one of the most important sources of contamination in marine environments as well as a serious threat to fisheries and aquaculture-based industries in coastal areas. Among these biotoxins Okadaic Acid (OA) is of critical interest as it represents the most predominant Diarrhetic Shellfish Poisoning biotoxin in the European coasts. Furthermore, OA is a potent tumor promoter with aneugenic and clastogenic effects on the hereditary material, most notably DNA breaks and alterations in DNA repair mechanisms. Therefore, a great effort has been devoted to the biomonitoring of OA in the marine environment during the last two decades, mainly based on physicochemical and physiological parameters using mussels as sentinel organisms. However, the molecular genotoxic effects of this biotoxin make chromatin structure a good candidate for an alternative strategy for toxicity assessment with faster and more sensitive evaluation. To date, the development of chromatin-based studies to this purpose has been hampered by the complete lack of information on chromatin of invertebrate marine organisms, especially in bivalve molluscs. Our preliminary results have revealed the presence of histone variants involved in DNA repair and chromatin specialization in mussels and clams. In this work we use this information to put forward a proposal focused on the development of chromatin-based tests for OA genotoxicity in the marine environment. The implementation of such tests in natural populations has the potential to provide an important leap in the biomonitoring of this biotoxin. The outcome of such monitoring may have critical implications for the evaluation of DNA damage in these marine organisms. They will provide as well important tools for the optimization of their harvesting and for the elaboration of additional tests designed to evaluate the safety of their consumption and potential implications for consumer's health.

Contents

Abbreviations: ATM, Ataxia Telangiectasia Mutated; DSBs, Double Strand Breaks; DSP, Diarrhetic Shellfish Poisoning; HABs, Harmful Algal Blooms; miRNA, microRNA; OA, Okadaic Acid; PP, Protein Phosphatase; PTMs, Post-Translational Modifications.

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1. Ecological relevance and genotoxic potential of marine biotoxins

One of the most important sources of contamination threatening the marine environment is the presence of massive algal proliferations. Such blooms consist of large accumulations of algae including phytoplankton, macroalgae and colorless heterotrophic protists. Although human activities and the associated increase in nutrient loadings are likely to be the primary reason in bloom formation (Cardozo et al., 2007), natural events also convey a great relevance. Oceanic/estuarine circulation as well as river flow influences the abundance and distribution of plankton. Furthermore, the combination of physical (e.g., currents, upwelling, etc.) and chemical (e.g., salinity, nutrients, etc.) factors of these systems, coupled with the different life cycles and behaviors of some taxa, contributes to the formation of these blooms (Sellner et al., 2003; Hallegraeff, 2010). Among the different types of massive algal proliferations, Harmful Algal Blooms (HABs) represent the most serious threat to fisheries and aquaculture-based industries in coastal areas. There are, however, toxin-producing species that cause significant impacts at low population densities and do not discolor the water. Indeed, this is the case of *Dinophysis* species, causing HAB at densities as low as 100 cells/L (Sellner et al., 2003). During these episodes, large amounts of potentially harmful biotoxins are produced by phytoplankton species, being subsequently accumulated by several marine organisms (including fish, molluscs and crustaceans) and eventually entering the human food chain. Thus, the bioaccumulation of these biotoxins represents a very serious health problem for human consumers (Cardozo et al., 2007). Although a very small fraction of phytoplankton species (roughly 1.5%) is able to produce biotoxins (Hallegraeff, 1995), the economic losses, the resources affected, and the number of toxins and toxic species involved have increased dramatically during the last 30 years (Van Dolah, 2000; Anderson, 2009).

Marine biotoxins can be grouped into 6 categories depending on their effects on consumers and their chemical nature including: diarrhetic, neurotoxic, amnesic, paralytic, azaspiracid shellfish poisoning and ciguatera fish poisoning (Rossini and Hess, 2010). Diarrhetic Shellfish Poisoning (DSP) toxins are the most important across European coasts (Aune and Yndestad, 1993), having already produced numerous toxic incidents (Villar-Gonzalez et al., 2007). The main active principle responsible for DSP is Okadaic Acid (OA) and the dinophysistoxins (DTX1, DTX2) (Vale, 2010), which are produced by dinoflagellates of the genera *Dinophysis* and *Prorocentrum* (Yasumoto et al., 1980; Naves et al., 2006) and represent the most predominant DSP biotoxin in Europe (James et al., 2010). Given that the ingestion of as few as 36–40 μg of OA already induces alterations in the gastrointestinal system causing nausea, vomiting, diarrhea and abdominal pain (Berven et al., 2001), specific normative has been applied by the European Union to guarantee the safety of consumers and public health (Regulation(EC), 2004), however small quantities of OA may be present in molluscs that have passed legal controls before its marketing, and therefore chronic exposure to this toxin may exist in regular consumers.

1.1. Molecular routes to OA damage in the genome

OA has been identified as a potent tumor promoter and apoptosis inducer (Suganuma et al., 1988) encompassing critical aneugenic and clastogenic genotoxic effects on the hereditary material (summarized in Fig. 1) in a cell line- and concentration-dependent manner (Valdiglesias et al., 2010; Valdiglesias et al., 2011a, 2011b). Furthermore, the small size and hydrophobic nature of this molecule (compared with other biotoxins such as microcystins) facilitates the diffusion of OA into different cell types and its interaction with cellular components (Xing et al., 2008). At the molecular level, OA specifically inhibits the Serine/Threonine Protein Phosphatases 1 (PP1) and 2A (PP2A) in mammalian model systems (Bialojan and Takai, 1988),

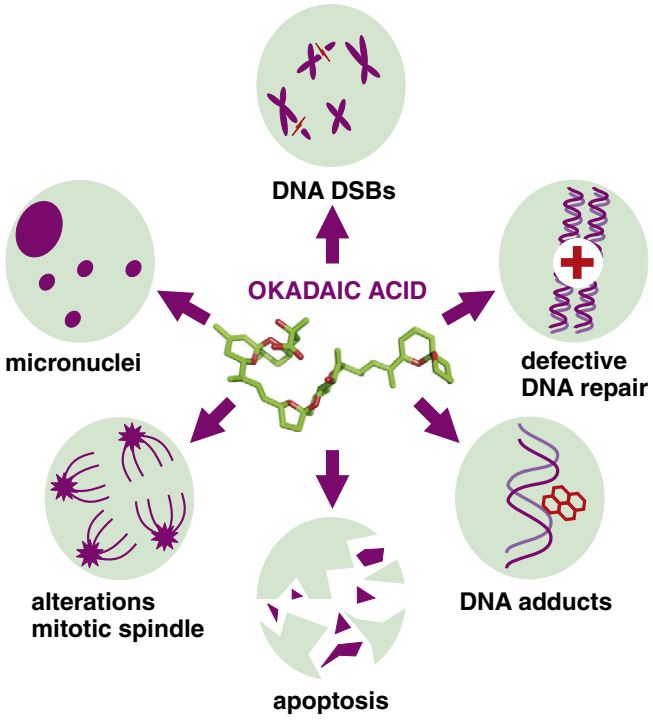

Fig. 1. OA has critical genotoxic effects, including DNA strand breaks (Traore et al., 2001; Valdiglesias et al., 2010), DNA damage and alterations in DNA repair (Traore et al., 2001; Valdiglesias et al., 2010), 8-OH-deoxyguanine adducts (Fessard et al., 1996), apoptosis (Valdiglesias et al., 2011c), alterations in the mitotic spindle (Van Dolah and Ramsdell, 1992), and micronuclei formation (Le Hegarat et al., 2003; Carvalho et al., 2006).

interfering with the myriad of processes involving these enzymes. For instance, several studies have demonstrated that OA causes cytoskeletal disruption, triggering apoptosis and membrane permeability alterations, among other effects (Leira et al., 2001). Furthermore, DNA oxidative damage has been also described in mammalian cell lines exposed to this biotoxin (Xing et al., 2008), as well misregulation of genes involved in critical cellular pathways (i.e., p53).

However, to completely understand the harmful effect of OA on the hereditary material it is important to consider that DNA is associated with proteins within the eukaryotic cell nucleus, forming a complex known as chromatin (van Holde, 1988). The fundamental packaging subunit of chromatin, the nucleosome core particle, consists of approximately 146 bp of DNA wrapped around a protein core composed by eight histone proteins, and is a highly dynamic nucleoprotein complex (Zlatanova et al., 2009). Chromatin must alter its conformation to counteract the multifaceted genotoxic effects of OA, mediating the activation of a plethora of mechanisms involved in the maintenance of genome integrity, most importantly transcription, DNA replication, recombination and repair (Moggs and Orphanides, 2004). This process (often called chromatin remodeling) requires the concerted action of histone-modifying enzymes, ATP-dependent chromatin remodeling complexes as well as histone variants with specialized functions (Ausió, 2006). The resulting histone marks, in combination with the specialized domains imparted by histone variants, dynamically modify the physical properties of individual nucleosomes and higher-order chromatin structures (Campos and Reinberg, 2009) in what it has been referred to as the 'histone language' based on a 'histone code' (Strahl and Allis, 2000).

1.2. Dynamic chromatin answers OA genotoxic effect within the cell nucleus

Among the different genotoxic effects OA conveys on chromatin, DNA Double Strand Breaks (DSBs) stand out as the most severe due

to the disruptive effect they produce on both DNA strands, eventually leading to the loss of genetic material (Altaf et al., 2007). Consequently, quick repair is required in order to prevent further damage to the cell, with mechanisms that involve the dynamic remodeling of chromatin in the earliest response (Fig. 2A). The role played by the H2A.X histone variant in response to DNA DSBs falls within this category. Accordingly, H2A.X histones of extensive regions flanking a damaged site become reversibly phosphorylated at their C-terminal SQEY motif (γ-H2A.X) creating the so-called 'H2A.X *foci*', which constitutes the primary signal activating the mechanism of DNA DSB repair within the cell nucleus (Li et al., 2005; Dickey et al., 2009). Once the repair process has been completed, the dissolution of the *foci* can occur following two different pathways: the first option involves γ-H2A.X dephosphorylation by phosphatases including PP1, PP2A, PP4, PP6, and Wip1 (Wild-type p53-induced phosphatase 1) (Freeman and Monteiro, 2010). The second option would lead to the release of γ-H2A.X of the nucleosome by ATP-dependent remodeling factors with the participation the histone variant H2A.Z (Altaf et al., 2007).

Although the phosphorylation and replacement of histone H2A.X constitutes the most widely studied chromatin-based mechanism of DNA repair, additional Post-Translational Modifications (PTMs) and histone variants have been linked to the response to DNA damage. For instance, dynamic phosphorylation of histones H2B (Fernandez-Capetillo et al., 2004), H3 (Prigent and Dimitrov, 2003), H4 (Utley et al., 2005) and H1 (Konishi et al., 2003; Kysela et al., 2005) has been shown to participate in the repair process (Fig. 2A). Phosphorylation, in combination with other PTMs such as acetylation (Bird et al., 2002; Tamburini and Tyler, 2005), may work as recognition signals for different protein complexes involved in DNA repair (Houben et al., 2007). Interestingly, recent studies also suggest that monoubiquitination is induced upon DSBs and plays a critical role in H2A.X phosphorylation by recruiting active Ataxia Telangiectasia Mutated (ATM) kinase to damage sites (Wu et al., 2011). Furthermore, different reports have directly or indirectly suggested the participation of variants other than H2A.X in the maintenance of genome integrity. For instance, the exchange of γ-H2A.X with H2A.Z seems to facilitate the recruitment of DNA repair factors and checkpoint factors (Krogan et al., 2004; Kusch et al., 2004; Mizuguchi et al., 2004). In addition, it is quite possible that histone H3.3 variant has also an active participation in the DNA repair process, as phosphorylation of this histone has a critical role in the regulation of chromatin accessibility to several factors and in chromatin dynamics (Hake et al., 2005). Furthermore, a

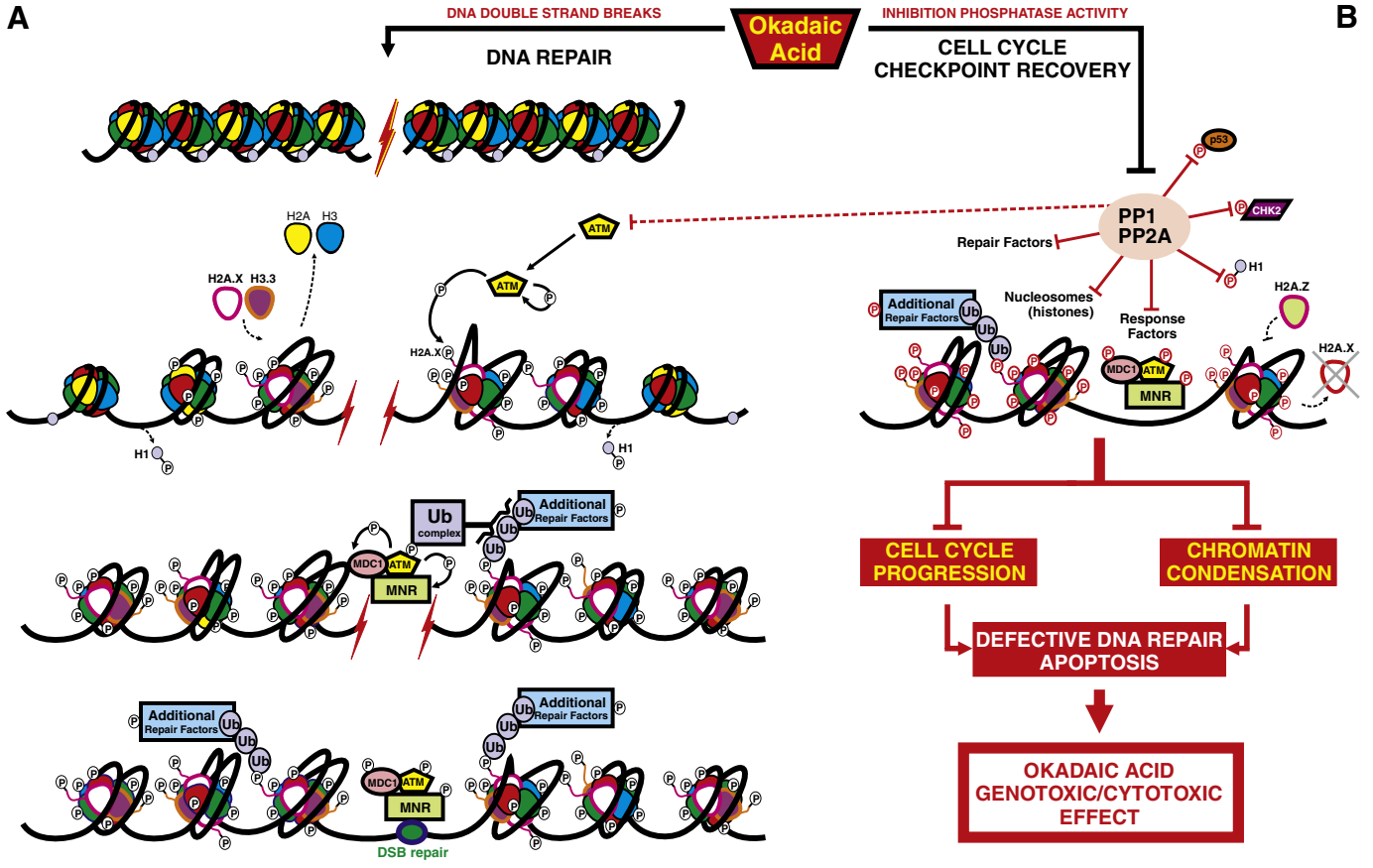

Fig. 2. OA induces DNA damage and interferes with the process of DNA repair. (A) Upon DSB damage H2A.X histones are immediately recruited into damaged regions, becoming reversibly phosphorylated (γ-H2A.X) by the Ataxia Telangiectasia Mutated (ATM) Serine/Threonine kinase (previously activated by autophosphorylation due to inhibition of PP1 and PP2A activity). During this process chromatin must be decondensed in order to provide access of repair factors to DNA, involving phosphorylation of histones H1 and H3.3. Although the present figure focuses on phosphorylation, other histone PTMs also participate in this process, including acetylation, methylation, ubiquitination and proline isomerization. Subsequently, γ-H2A.X acts as recognition signal for different repair factors. Firstly, the MRN complex (Mre1, Rad50 and NBS1 proteins) binds and keeps DNA ends close, while the Mediator of DNA damage Checkpoint protein 1 (MDC1) complex binds to γ-H2A.X stabilizing the MRN complex and retaining multiple checkpoint and adaptor proteins. Later on, ATM phosphorylates Mre1 and MDC1 forming a docking place for the union of the ubiquitination complex which, by adding several ubiquitin molecules to γ-H2A.X, mediates the recruitment of additional repair factors involved in proper chromatin repair (Oberle and Blattner, 2010). The repair process is then completed by the ligation of free DNA ends. (B) Once the DSB has been repaired, the machinery must be disassembled and the chromatin structure condensed in order to resume cell cycle. However, this process is going to be impaired by the effect of OA inhibiting PP1 and PP2A activity and preventing the dephosphorylation of the components of the repair machinery (including γ-H2A.X, H1, H3.3, p53 and the checkpoint protein kinase Chk2, as well as several other repair factors) (Travesa et al., 2008; Oberle and Blattner, 2010). Furthermore, OA will interfere with H2A.Z expression, hampering its replacement for H2A.X. As a result, OA will promote defective DNA repair, eventually leading to cellular apoptosis.

new dimension in the regulation of DNA damage response has been recently ascribed to microRNAs (miRNAs), including a repressive role of miRNA-138 on H2A.X expression (Hu and Gatti, 2011; Wang et al., 2011).

2. Biomonitoring of harmful biotoxins in the marine environment

The contamination of coastal areas with OA resulting from HABs has drastic negative effects for both the economy of aquaculture-based industries and the health of human consumers exposed to intoxication. Such a harmful effect is especially evident in the case of bivalve molluscs harvested in estuarine areas, which are completely exposed to OA given their sessile and filter-feeding life style (Cajaraville et al., 2000; Franzellitti et al., 2010). More specifically, mollusc production has been reduced 25% during the last 5 years as a consequence of HABs in coastal areas from Galicia (NW Spain), where this industry maintains 11,500 direct jobs and a net worth of 115 million euro/year (FAO, 2011), representing one of the major driving force for the economy of that region.

A very important effort has thus been dedicated to analyze the effect of OA on marine organisms during the last two decades, especially through biomonitoring programs that use mussels as sentinel model organisms (Wells et al., 2001). However, the reliance of such analyses on physicochemical and physiological parameters often results in an indiscriminating low sensitivity, especially for the monitoring of long-term exposure to trace levels of OA (Valdiglesias et al., 2011a). Consequently, and given the molecular basis of the genotoxic effect of OA, the development of molecular probes focusing on the effects of this biotoxin at the level of chromatin, should provide a very appealing alternative in order to overcome this problem. Indeed, the power of chromatin-based genotoxicity tests has been already demonstrated in mammals using H2A.X phosphorylation as biomarker for DNA repair following exposure of cells to suspected DNA-damaging compounds such as cigarette smoke, polycyclic aromatic compounds, and crude oil among others (Ibuki et al., 2007; Albino et al., 2009; Dickey et al., 2009; Mattsson et al., 2009; Watters et al., 2009).

2.1. Beams and nails of chromatin knowledge in bivalve molluscs

The development of chromatin-based gentoxicity tests constitutes a feasible goal in model organisms such as human and mouse, for which an important body of knowledge pertaining chromatin-associated factors, remodeling mechanisms and histone variants has been amassed during the last 25 years (Ko et al., 2008; Srivastava et al., 2009; Talbert and Henikoff, 2010; Zhou et al., 2011). Nonetheless, such information is very limited or absent in many non-mammalian organisms. Molluscs are not an exception to this, making a difficult access to the structural and metabolic processes of chromatin in these organisms and by default to their potential application in the study of genotoxicity. During the last decade, work from our research group has started to fill this gap by studying chromatin in bivalve molluscs (Eirín-López et al., 2009). As a result, histone multigene families from several species (including mussels, clams and razor clams, among others) have been widely characterized (Eirín-López et al., 2002, 2004b; González-Romero et al., 2008, 2009). Our evolutionary analyses have shown that these families evolve subject to a process known as birth-and-death evolution, which is responsible for the genetic diversification observed among histone family members (Eirín-López et al., 2004a; González-Romero et al., 2008; González-Romero et al., 2010).

However, it remained to be demonstrated whether histone variants with dedicated functions were already evolutionary differentiated in this group of organisms and if so, how could they be used in genotoxicity studies. Our most recent unpublished studies on this topic strongly suggest that the answers to both questions are in fact affirmative. Firstly, transcriptomic analyses carried out on different species of bivalves have provided evidence for the presence of genes encoding histone variants H2A.X, H2A.Z and H3.3 (manuscript in preparation). These genes are transcribed and translated into protein products that exhibit a high extent of similarity with those found in chordates, suggesting their participation in similar functional roles. Secondly, screening of OA-specific expression libraries from mussels has revealed that at least H2A.Z is specifically downregulated in response to harmful levels of OA, pointing toward its involvement in the maintenance of genomic integrity in response to this biotoxin (manuscript in preparation). Such hypothesis is supported by the ability of mussel H2A.Z to dynamically affect chromatin structure, as we have evidenced by preliminary nucleosome reconstitution experiments. This preliminary work has significant implications for the study of chromatin not only because it represents the first description of functionally differentiated variants in bivalve molluscs but also more importantly, as it provides the basis for an innovative and multidisciplinary exploration of the potential application of histone variants as biomarkers for genotoxicity.

2.2. Chromatin-based genotoxicity tests: a leap forward in the study of marine biotoxins

The genotoxic effects of OA will have their imprint on the processes involved in the maintenance of genome integrity. For one, its phosphatase-inhibitory activity will interfere with signaling mechanisms involved in DNA repair and apoptosis (Bialojan and Takai, 1988). Also, OA will affect the chromatin metabolism processes (recruitment of histone variants and associated PTMs) related with genome integrity. Indeed, it has been demonstrated that OA causes a significant reduction in DNA repair and cellular viability (Chowdhury et al., 2005), as well as defects in cell cycle checkpoint recovery as indicated in Fig. 2B (Carlessi et al., 2010). Thus, the development of molecular assays using histone variants as biomarkers will represent a leap in the study of OA genotoxicity in bivalve molluscs due to their high sensitivity and ability to detect early response to DNA damage. By defining the cause-and-effect relationship between OA exposure and the activation of apoptosis and DNA repair mechanisms, these tests will set up a pattern that could be applied on natural populations and will be of an outmost interest for the conservation and health reasons outlined earlier.

An important part of the specialization imparted by H2A.X, H2A.Z and H3.3 to chromatin is involved in the maintenance of genome integrity and transcriptional regulation (Ausió, 2006; Eirín-López and Ausió, 2007). Therefore, a first stage in assessing the potential of these variants as genotoxicity biomarkers will require the characterization of the structural constraints that lead to the specific function of these variants in nucleosomes from bivalve molluscs (Fig. 3A). However, the analysis of PTMs affecting these variants in response to DNA damage (i.e., immunodetection of phosphorylation of terminal Serine in H2A.X and Lysine acetylation in H2A.Z) will also be critical for the assessment of the genome-wide distribution of the chromatin marks involved in this process, especially as it pertains to DSB lesions. A second stage in the development of chromatin-based genotoxicity tests should be framed around the study of the mechanisms specifically involved in the response to OA. To this end, the comparison of transcriptomes obtained from individuals exposed to increasing concentrations of OA may represent a very powerful tool in defining candidate genes involved in the molecular response to this biotoxin, especially for those of histones with a potential role in DNA repair (Fig. 3B). As mentioned above, this approach has already produced information regarding the downregulation of H2A.Z in response to high OA concentrations (manuscript in preparation). Its further development will be very relevant at the time of preparing powerful analytical tools such as microarrays designed for the rapid and efficient diagnostic identification of genes involved in the OA response.

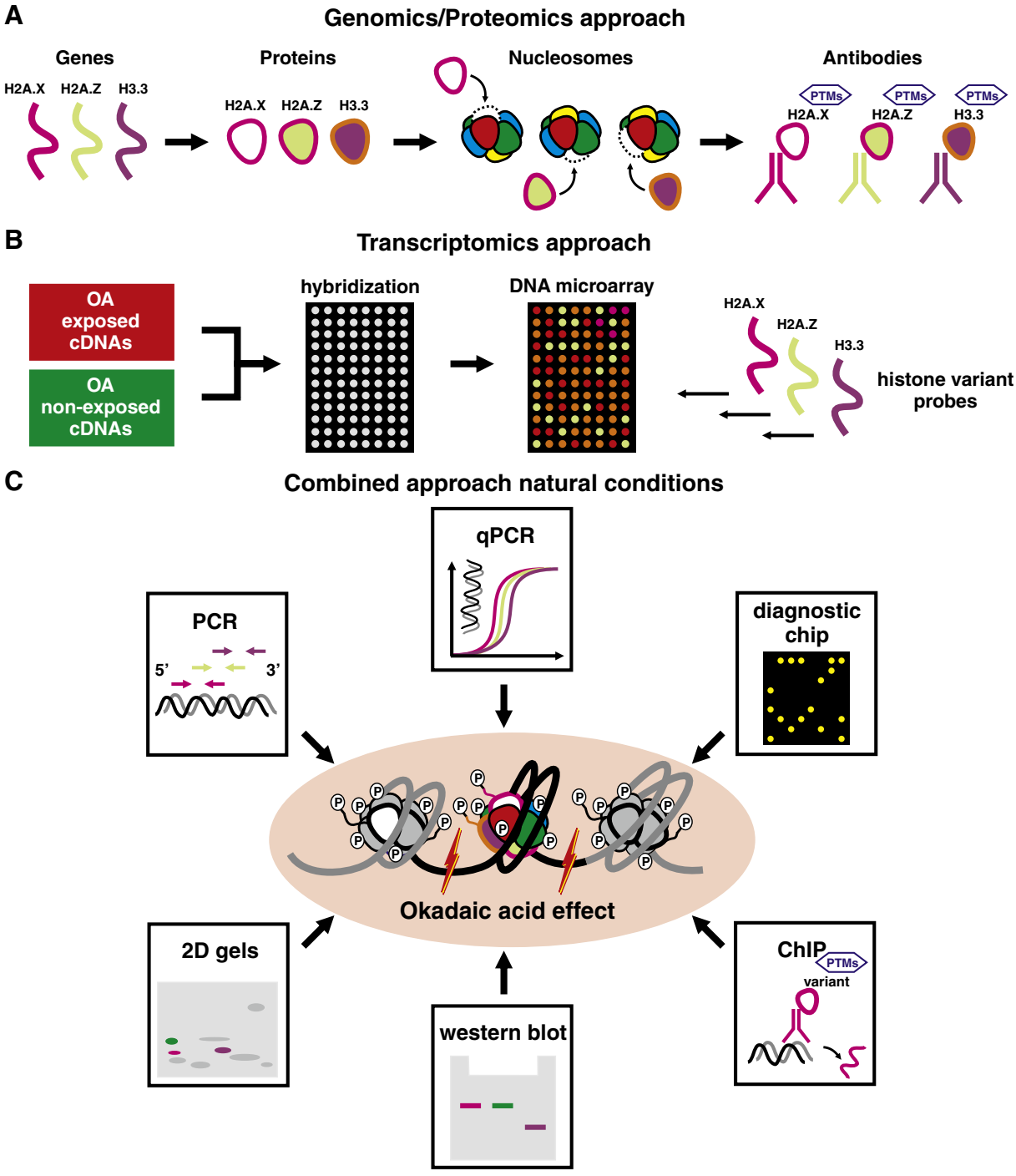

Fig. 3. The implementation of chromatin-based tests of OA genotoxicity in natural populations of molluscs will require previous knowledge on chromatin structure and dynamics in these organisms. (A) By genomic/proteomic approaches, histone variants and their associated Post-Translational Modifications in response to OA will be characterized, helping to decipher the kind of specificity they impart to the nucleosome particle. This strategy will bring the opportunity to synthesize specific antibodies against modified variants, allowing their immunolocalization and the chromatin immunoprecipitation (ChIP) of genomic regulatory regions. (B) Additionally, the expression profiles of genes differentially regulated as a consequence of OA exposure will be revealed through transcriptomic analyses. The mining of the resulting data will also reveal differential regulation of target histone variants. (C) The evaluation of the genotoxic effect of OA in natural populations will rely in the combination of both approaches, allowing to: isolate histone variants and quantify histone gene expression levels (PCR, qPCR), perform diagnostic identification of histone variants upregulated/downregulated in response to OA (diagnostic chip), determine Post-Translational Modifications involved in OA response (western blots and mass spectrometry), as well as characterize the regulatory regions where these variants are potentially deposited (ChIP).

The final purpose of developing chromatin-based genotoxicity tests seeks to evaluate the effect of OA in natural populations of bivalve molluscs. However, this would require a previous set up of the experimental conditions in the lab, including the analysis of H2A.X, H2A.Z and H3.3 expression levels (in response to increasing OA concentrations) as well as the immunodetection and quantification of PTMs associated with DNA repair (especially phosphorylation of H2A.X). The expectation being that the outcome of these experiments will shed light into the potential cause-and-effect relationship between OA exposure and the response of chromatin metabolism, which would represent the basis for the application of these tests to natural populations in the marine environment (Fig. 3C).

3. Concluding remarks

The genotoxic effects of different pollutants are mirrored by alterations of chromatin metabolism, including DNA repair and replication, regulation of gene expression and cell division, among others. Consequently, the development of chromatin-based tests for detecting and evaluating the genotoxic effect of biotoxins represents an important advance in the biomonitoring of pollution in the marine environment. Compared with more traditional approaches based on the biomonitoring of physiological parameters, the implementation of such tests have the potential for an earlier and more sensitive way of detection of the genotoxic effects of biotoxins such as OA. The resulting subsequent improvement will help design new strategies of evaluation of DNA damage, optimization of harvesting techniques and an enhancement of the quality controls used to monitor and ensure consumer's health.

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